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ON THE VALUE OF AN ANTIBIOTIC SUBSTANCE OF BACTERIAL ORIGIN IN THE TREATMENT OF SEED

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ON THE VALUE OF AN ANTIBIOTIC SUBSTANCE OF BACTERIAL ORIGIN IN THE TREATMENT OF SEED

J. J. Goodman

Department of Plant Science

A THESIS

submitted to the University of Alberta
in partial fulfilment of the
requirements for the degree of

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ON THE VALUE OF AN ANTIBIOTIC SUBSTANCE OF BACTERIAL ORIGIN IN THE TREATMENT OF SEED

J. J. Goodman

INTRODUCTION

The subject of microbial antagonism is almost as old as microbiology itself. Many reviews have been written on the whole subject, the most recent and complete one by Waksman (36). Hoogerheide (15) has reviewed antibiotic substances produced by soil bacteria.

The work reported here resolves itself into two main sections. The first deals with the isolation and purification of an antibiotic from a soil bacillus and with factors affecting its production. The second describes work done on the use of this antibiotic in the control of a seed-borne plant pathogen, namely *Xanthomonas translucens* (J. J. and R.) Dowson, which causes Bacterial Blight of barley. No general literature review will be given, but pertinent references will be cited throughout.

THE ANTIBIOTIC SUBSTANCE

SOURCE OF THE ANTIBIOTIC

Source of Isolates

Soil has been a fruitful source of antagonistic microorganisms and was largely employed as such in this study. Serial dilutions up to 1:100,000,000 of Edmonton black soil were plated out on potato-dextrose agar. Plates were then sprayed with a water suspension of one of two organisms: Xanthomonas translucens f.sp. hordei-avenae (J. J. and R.) Dowson emend. Hagb., hereinafter referred to as Xan. 190; and the second, a culture of Pseudomonas coronafaciens (Elliot) Stapp, used by S. Goto (11). Soil organisms showing antagonism to either of the test organisms, as evidenced by a sterile halo around them, were isolated and kept in pure culture on potato-dextrose agar slants. In all, forty more or less antagonistic organisms, including four Actinomycetes, eight Penicillia, and the remainder bacteria, were isolated.

Preliminary Choice of Isolates

To narrow down the number of isolates, a preliminary selection was made on two bases: (1) on the antagonism to Xan. 190, and (2) on the phytotoxic effect.

For the first, the isolates were streaked on potato-dextrose agar plates and allowed to incubate at 25°C. until some growth was made. The plates were then sprayed with a water suspension of Xan. 190 and allowed to incubate for several days. The width of the sterile zone around the isolate was taken as a measure of its activity. Since most of the isolates were originally chosen on this basis the majority of them were active to a greater or lesser extent.

Two methods of determining the phytotoxic effect of the isolates were used. In the first, seeds of wheat, oats, and barley, were placed between two sheets of filter paper in a Petri plate. Ten seeds were used per plate. Three ml. of 5% bran-extract medium, in which the isolate had been growing for nine days, were then added to the Petri plate along with 3 ml. of water. The plates were incubated at 25°C. for three days, after which notes on germination and average shoot length were taken. According to the second method, the isolates were streaked on potato-dextrose agar in plates containing 15 ml. of agar, and incubated at 25°C. for four days. Twenty-four-hour-old, sterile seedlings

of wheat and acid hulled barley were then placed on the agar 10 to 15 mm. away from the growing colony. Five seedlings of wheat were placed on one side and five of barley on the other side of the colony. After 48 hours the seedlings were observed for growth and for root abnormalities.

On the basis of antagonism to Xan. 190 and the preliminary phytotoxic tests, five isolates were chosen for further study*.

Selection of A32

It was soon realized that we could afford to concentrate our efforts on only one of the five isolates. The criterion of choice was to be the effect of staled medium--i.e. medium in which the isolate had grown--on the germination of surface-sterilized wheat and its effect on the root length of the subsequent seedlings. The five isolates were grown for several days on a 5% bran-extract medium. The staled medium was then autoclaved for 10 minutes and made up to various dilutions with sterile distilled water. Ten ml. of each dilution were added to Petri plates fitted with filter paper and containing 50 seeds each.

---

* The isolation and preliminary work on the various isolates was done by Mr. A. W. Jackson whose direction and help in the early part of the project are gratefully acknowledged.

The plates were incubated at 25°C. for three days, after which the germination and the length of the longest root on the germinated seeds were recorded. The results are presented in Table I.

The figures given in Table I do not present the complete picture since many of the seedlings germinated, yet did not have measurable roots. This is especially the case where undiluted staled medium was used. The number of seedlings with measurable roots in the undiluted series was as follows:

Isolate	Number of seedlings with measurable roots
A32	31
A47	16
A45	16
A61	4
A65	3

These results, taken together with those in Table I, show that A32 is relatively non-phytotoxic. To confirm this, a test similar to the first one was carried out using unsterilized staled A32 medium only. Both root and stem length, as well as the germination, were measured. The results are presented in Table II.

TABLE I

Percent germination and average length in mm. of longest root of wheat seedlings grown for 3 days in the staled medium of 5 isolates

Staled medium from isolate	Dilution													
	0		1/10		1/25		1/50		1/100		1/250		1/500	
	% ger.	Root length	% ger.	Root length	% ger.	Root length	% ger.	Root length	% ger.	Root length	% ger.	Root length	% ger.	Root length
Unstaled medium	95	12.0	99	26.5	97	25.7	99	29.6	99	31.0	99	30.0	98	27.0
A32	94	6.5	98	22.0	--	--	100	30.0	100	33.0	72	30.0	100	30.0
A47	94	11.0	100	16.4	100	20.5	98	24.0	100	24.6	100	28.0	--	--
A45	88	7.5	98	23.0	98	27.0	98	26.7	98	30.0	100	34.7	--	--
A61	94	7.0	100	16.0	98	19.0	100	27.0	98	27.0	100	31.0	--	--
A65	88	5.0	100	14.0	100	33.0	98	28.0	100	31.0	96	31.5	--	--

TABLE II

Percent germination and average root and stem length in mm. of wheat grown in unsterilized staled A32 medium

Dilution	% germination	Root length	Stem length
H_2O (check)	70	44.1	25.3
1/10 unstaled medium	63	50.0	25.4
Whole staled medium	66	25.4	20.9
1/10 staled medium	69	31.5	22.0
1/50 " "	70	40.6	24.3
1/100 " "	57	35.9	22.8
1/250 " "	66	39.0	22.0
1/500 " "	68	41.0	26.0

The results of the second test confirm the non-phytotoxic effect of A32. This, coupled with the fact that it was very active against Xan. 190, led to its being chosen as the isolate for detailed study.

Identification of A32

A32 is a Gram-positive, obligately aerobic, spore-forming bacillus. It grows as single rods or sometimes in chains. Cells are 2.4μ to 3.9μ by 0.4μ to 0.6μ. Spores

are ellipsoidal, central to sub-central, 1.2μ to 1.8μ by 0.6μ. After incubation for 18 hours at 35°C. on potato-dextrose agar, it forms an abundant, dull, opaque, brownish colony with a rough, ground-glass-like topography. The growth over the slant is flat and fairly adherent. Gelatin is liquefied after four days at room temperature. Hydrogen sulphide production, methyl-red, and indole tests are negative. Voges-Proskauer test is positive. Starch hydrolysis is positive. No gas is produced. pH values after 4 days' incubation at 35°C. indicate acid production from glucose, maltose, fructose, mannose, nutrose, and probably from mannitol (21). These biochemical characteristics place A32 in the *B. subtilis* group. It was later identified by Dr. N. R. Smith of the U.S.D.A. as a typical rough strain of *Bacillus subtilis*. Through the National Research Council a culture of *B. subtilis* N. R. Smith No. 231, used in the production of subtilin (17) was obtained. A comparison between A32 and No. 231 is given in a subsequent section.

Since A32 is *B. subtilis* it is not surprising that it shows antibiotic properties. Certain strains of this species have been known for many years to be antagonistic to a number of pathogenic and non-pathogenic bacteria and fungi. The early literature is given in (17). Olivier (26) suggests that the biological properties of *B. subtilis* may have clinical applications. The production of antibiotics

from *B. subtilis* as from other organisms is very dependent upon the strain of organism used. Jansen and Hirschmann (17) obtained no activity against any of the test organisms employed when they used a strain of *B. subtilis* other than their subtilin strain. Another antibiotic from *B. subtilis* was obtained by Johnson, Anker and Meleny (18), while still another antibiotic from a third strain was obtained by Foster and Woodruff (7). In order that comparisons may be made, it is important for the investigator to characterize the strain he is working with. It seems very evident from recent work that different strains of a given organism may be as fruitful a source of new antibiotics as is an entirely different species. In spite of the well known antagonistic property of *B. subtilis* no report has come to my attention of its use in the field of plant pathology.

FACTORS AFFECTING THE PRODUCTION OF THE ANTIBIOTIC OF A32

Methods of Assay

Early in the investigation the streak method noted above was used to assay the activity of the isolates. With the selection of A32 the agar-cup technique was used to

measure activity. This technique consists in removing a round block of agar from a Petri plate seeded with the test organism--in this case Xan. 190--by means of a flamed cork-borer. Into the "well" thus formed was pipetted a measured amount of the fluid to be tested. The activity was determined by measuring the size of the sterile zone or halo around the well. The method has drawbacks in that such plates need care in handling in order not to spill any of the fluid in the well; and there is also a great deal of opportunity for contamination both in the making of the well and in the pipetting of the liquid into it.

Early in the study, therefore, the paper-disc, agar-plate method of assay was adopted. The method was suggested by Vincent and Vincent (33). It has been found by deBeer and Sherwood (2) to give standard reproducible results comparable to those obtained by other methods. Filter paper discs 14 mm. in diameter were cut with a cork-borer from No.1 Whatman filter paper. These discs were then sterilized in the autoclave and allowed to dry before being used. Test plates were seeded, each with 1 ml. of a suspension of Xan. 190, in sterile distilled water. No attempt was made to standardize the number of organisms in the suspension, though it was kept relatively constant by use of a standard loopful of Xan. 190 in 10 ml. of water. To the 1 ml. of suspension Xan. 190 were added 10 ml. of melted, cooled nutrient agar.*

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* 3 gm. Difco Beef extract, 5 gm. Difco peptone, 15 gm. agar, 1000 ml. distilled H_2O.

ge) from a [illegible] plate [illegible]
—in this case Kan. 150—by means of a [illegible]
into the "well" thus formed was [illegible]
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ing in order not to spill any of the fluid in the
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the the number of organisms in the suspension, but
nt relatively constant by use of a standard [illegible]
[illegible] in 10 ml. of water. In the 1 ml. of [illegible]

The agar was kept in tubes in 10-ml. lots and was melted and poured into the plates just before testing. After the cooled agar was added to the plates they were swirled around to distribute the test organism and then set aside to harden. Immediately the agar had sufficiently hardened the discs were applied. A disc was grasped with a pair of pointed forceps and dipped into the solution to be tested. Excess solution was allowed to drain off by touching the disc against the side of the vessel. The disc was then placed on the surface of the agar. Four discs were thus placed in each plate, though in some cases three were used. The forceps were flamed between the application of each disc and the cover of the test plate was lifted just high enough to allow the disc to be inserted. The plates were then incubated at 30°C. for 18 to 24 hours. The width of the halo was measured to the nearest mm. and this gave a measure of the activity of the fluid tested (Figure 1). Certain additional factors were found to affect the size of the halo. These are discussed separately later. To test the activity of solids, powders, etc., similar plates were prepared. Bits of the material were picked up on the end of a flamed loop and placed on the surface of the agar, or drawn across it in the form of a cross. This gave a qualitative and a rough quantitative test for solid material, which was all that was desired in most cases. Mention will be made in the text where the method of

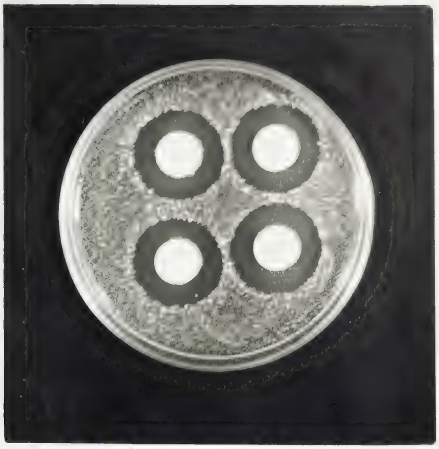

Figure 1

Type of halo normally obtained with the agar-plate paper-disc technique using Xan. 190 as the test organism

assay was varied.

Effect of Various Media on the Growth and Antibiotic Activity of A32

Bran Extract Media

In the early part of the investigation a 5% bran-extract medium was used. This medium was made as follows: to 50 gm. of bran was added enough distilled water to make up 1 litre. This mixture was then autoclaved for 10 minutes at 15 lb. pressure. The resultant mash was then filtered through several layers of cheese-cloth and the liquid made up to 1 litre. Lots of the medium were then dispensed in shallow layers into Erlenmeyer flasks and sterilized for 20 minutes at pressure. Bran medium was found to produce a thick pellicle of growth of A32 when incubated 3 to 4 days at 35°C., and proved very favorable for the production of antibiotic activity.

To see whether various carbohydrate and nitrogen compounds added to the bran-extract medium would improve it for antibiotic production, the following were added separately to the medium:

Nitrogenous substances added at the rate of 0.1%	Carbohydrate added at the rate of 0.5%
$NaNO_3$	Dextrose
$(NH_4)_2SO_4$	Maltose
Urea	Sucrose
Difco Peptone	Lactose
Difco Tryptone	Fructose
Difco Yeast Extract	Galactose

Check

The flasks were inoculated and allowed to incubate at 35°C. for 5 days. Their contents were then tested for activity. None of the above materials added singly increased the activity greatly, though there were slight differences. The materials containing organic nitrogen increased the activity most, with Tryptone appearing to be the best in this respect. Of the added sugars, sucrose gave the best results.

The effect of concentration of bran in the medium was also tested. A 2.5%, 5%, 8%, and 10% bran-extract medium, with Tryptone at the rate of 0.1% and sucrose at the rate of 0.5% added, was tested for production of activity. There was a definite increase in activity as the concentration of the bran extract increased, with 10% giving the highest activity. For most of the work with bran-extract media, however, the 5% concentration was employed.

Corn-Steep-Liquor Medium

A32 was found to produce good growth on a 5% corn-steep-liquor solution adjusted to a pH of 6.5 to 7. Such a staled medium, when tested, was found to have no activity against Xan. 190. The addition of Dextrose at 0.5 gm./100 ml. to this medium resulted in growth and activity. It would appear that the amount of growth does not affect the production of the antibiotic. This will be brought out more clearly in tests described below.

Czapek-Dox Corn-Steep-Liquor Medium

This medium taken from Burkholder and Sinnot (5) consisted of the following:

$NaNO_3$	2.0 gm.
KH_2PO_4	1.0 gm.
KCl	0.5 gm.
$MgSO_4.7H_2O$ ...	0.5 gm.
$FeSO_4$	0.01 gm.
Dextrose	40 gm.
Corn Steep Liquor	20 gm.

Distilled water to make up 1 litre

This medium encouraged the growth and the activity of A32.

Media containing corn-steep-liquor were rather difficult to handle because of the necessity of pH adjustment,

and also because of the viscous nature of the liquor. For the extraction studies too, a chemically defined medium was more desirable than bran-extract media and those containing corn-steep liquor.

Chemically Defined Media

Media used in the production of tyrothricin (6), streptothricin (35), and subtilin (17) were slightly modified and tried for growth and production of the antibiotic activity of A32. The media were made up as follows:

1. Modified tyrothricin medium
    - Gelatin - 1%
    - $MgSO_4.7H_2O$ - 0.05%
    - KH_2PO_4 - 0.2%
    - NaCl - 0.5%
    - Difco Yeast Extract - 1 gm.
    - Distilled water
2. Modified streptothricin medium
    - Dextrose - 10 gm.
    - Tryptone - 5 gm.
    - K_2HPO_4 - 0.5 gm.
    - NaCl - 0.5 gm.
    - $FeSO_4$ - 0.1 gm.
    - Distilled water - 1000 ml.

3. Modified subtilin medium

$MgSO_4 \cdot 7H_2O$ - 0.5 gm.
KCl - 0.5 gm.
K_2HPO_4 - 1.0 gm.
Dextrose - 30 gm.
Asparagine - 0.3%
Distilled water - 1000 ml.

Fifty ml. of medium were dispensed in each of five 250 ml. Erlenmeyer flasks and sterilized. A32 was then added. Notes on growth were recorded on the third and sixth days of incubation at 35°C. The activity of the staled medium was tested on the sixth day. The results were as follows:

Tyrothricin medium: There was only a thin pellicle of growth on both the third and sixth days. The average activity* against Xan. 190 was 4.5 mm.

Streptothricin medium: There was a thin pellicle of growth not quite over the whole surface of the medium on the third day. On the sixth day the pellicle was thick and wrinkled, but still had not covered the whole surface. The activity against Xan. 190 was 9.5 mm.

Subtilin medium: The growth was thick and wrinkled on the third and sixth days. The activity against Xan. 190 was 11.5 mm.

The two media showing the most activity were each bulked and extracted by the n-butanol method (discussed under

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* Average activity is the average of 4 discs for each of the 5 flasks of medium.

Extraction Studies). The staled streptothricin medium yielded a butanol extract of 13 mm. activity, and the staled subtilin medium one of 13.5 mm. The n-butanol method made a complete job of extracting the staled subtilin medium but not of the streptothricin medium. On the basis of activity produced and ease of extraction, it was decided to adopt the modified subtilin medium. It differs from the medium of Jansen and Hirschmann (17) only in the substitution of dextrose for sucrose and in its lack of trace elements.

Effect of Asparagine Content on the Activity Produced in the Modified Subtilin Medium

At the time this work was being done the supply of asparagine in the laboratory was quite low and it seemed desirable to know whether good growth and activity could be obtained in a medium of lower asparagine content; and also whether aspartic acid could be substituted. Flasks of modified subtilin medium containing different percentages of asparagine or aspartic acid were made up, sterilized, sown with A32, and incubated for several days at 35°C. Two flasks of each percentage were tested. The activity was measured in two test plates each with four discs, for each flask. The results, presented in Table III, are thus an average of 16 discs.

TABLE III

Growth and activity of A32 in a modified subtilin medium containing varying percentages of asparagine

Asparagine, %	Growth	Activity (in mm.) against Xan.190
0.0	0	0
0.005	0	0
0.01	+	4.5
0.02	++	7.5
0.03	++	7.5
0.04	++	7.4
0.05	+++	8.0
0.08	++	7.5
0.1	+++	8.4
0.15	+++	8.7
0.2	+++	8.4
0.25	++++	8.3
0.3	++++	8.0
Aspartic Acid		
0.02	++	8.1
0.05	++	8.5

In the results of the individual flasks it was noted that there was a close correlation between growth and activity only until a certain level of growth was reached, after which there did not seem to be much increase in activity. The results show that aspartic acid may be substituted for asparagine. For purposes of economy (at the time) the .03% level of asparagine was adopted for subsequent work. This medium will be referred to hereinafter as .03% asparagine medium. It has served very well for our purposes as long as there was

a good "catch" with enough growth to cover the surface of the medium.

Effect of Aeration of Medium on the Antibiotic Activity of A32

Since A32 is an aerobic organism, the amount of exposed area in relation to the volume of the medium--i.e. the aeration--would be expected to affect the activity. Most antibiotics are produced in shallow layers of medium, or else the medium is artificially aerated. It had been noticed that shallow layers of medium gave the highest activity with A32. Several tests were run in this connection.

Early in the investigation 100 ml. of 5% bran-extract medium were dispersed in Erlenmeyers varying from 125 to 1000 ml. capacity. The flasks were inoculated and incubated at 35°C. for four days. Though there was a similar amount of surface growth in all the flasks, there was greater activity in those with shallow layers of medium.

A more extensive test, using a .03% asparagine medium, was carried out. Different amounts of medium were dispersed in 250-ml. Erlenmeyer flasks as follows:

Flask no.	Amount of medium	Depth of medium
1	10 ml.	2 mm.
2	30 ml.	6 mm.
3	55 ml.	12 mm.
4	100 ml.	22 mm.

The flasks were inoculated and incubated at 30°C. for seven days, after which the activity of the cultures was tested. They were put back at 30°C. and the activity tested every day for 12 consecutive days. The results are presented in Table IV and shown graphically in Figure 2. They represent an average of 8 discs at each depth.

TABLE IV

Effect of depth of medium on activity (mm.)

Depth of medium	Age of culture in days											
	7	8	9	10	11	12	13	14	15	16	17	18
2 mm.	9.7	9.7	10.7	10.5	12.0	11.4	11.4					
6 mm.	7.6	9.1	10.2	9.9	11.2	9.7	9.7	10.9	11.4	11.5	10.1	10.7
12 mm.	6.1	6.7	8.4	7.2	10.6	9.7	9.7	10.9	10.6	10.9	9.7	10.4
22 mm.	4.4	4.5	4.7	6.0	7.2	5.9	5.9	6.9	6.4	7.0	9.2	9.6

Though these results are based upon only one flask at each depth, they clearly show that shallow layers of medium are more effective than deep ones. Had more flasks been used the minor day-to-day variations would very likely have been smoothed out. One objection to the foregoing test might be that different amounts of medium were used at each depth. It will be noted, however, that the 22-mm. series, which contained the greatest amount of medium, consistently

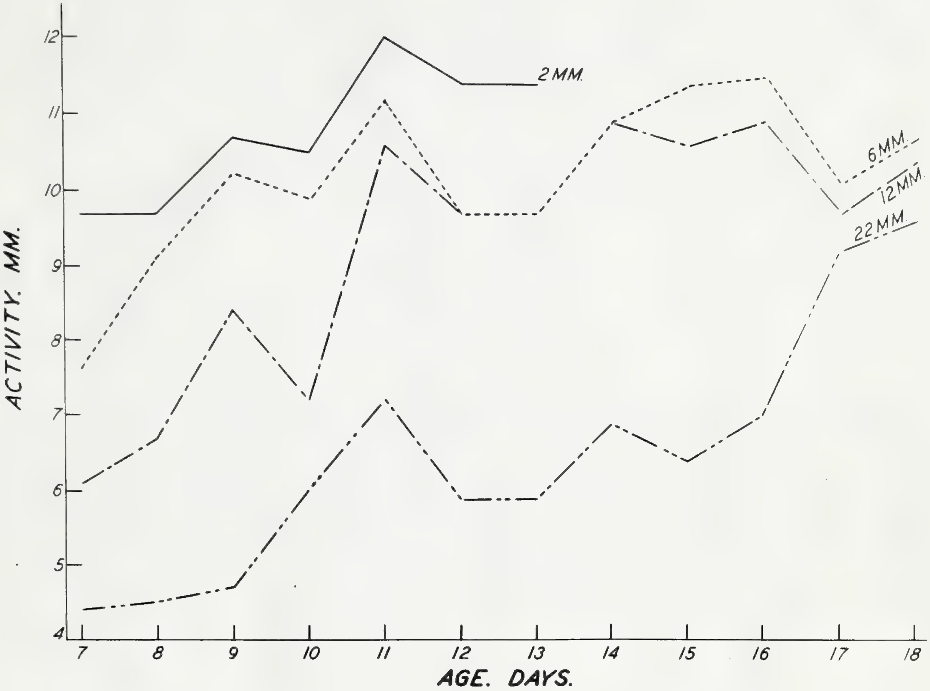

Figure 2

Effect of depth of medium on the activity of B. subtilis A32 cultures

gave the lowest activity.

A second test using a constant amount of medium was carried out. A .03% asparagine medium was dispensed in 100-ml. portions into a series of Erlenmeyer flasks of different sizes, as shown below:

Size of flask	Amount of medium	Depth
125 ml.	100 ml.	40 mm.
200 "	"	25 "
300 "	"	20 "
500 "	"	13 "
1000 "	"	10 "

Two flasks of each size were inoculated with A32 and were incubated at 30°C. for four days, after which readings of activity were begun. Each figure presented in Table V and in Figure 3 represents an average obtained from 16 discs.

TABLE V

Effect of depth of medium on activity (mm.)

Size of flask	Depth of medium	Age of culture in days							
		4	6	8	10	14	18	22	27
125 ml.	40 mm.	6.2	8.0	7.1	7.9	7.9	9.2	7.0	5.7
200 "	25 "	6.7	7.4	7.9	8.6	8.7	8.7	8.2	7.1
300 "	20 "	6.5	8.2	7.6	8.1	-	-	-	-
500 "	13 "	6.9	9.0	9.2	9.9	9.8	9.8	8.6	7.4
1000 "	10 "	9.2	9.5	9.6	10.7	10.6	11.4	9.7	9.9

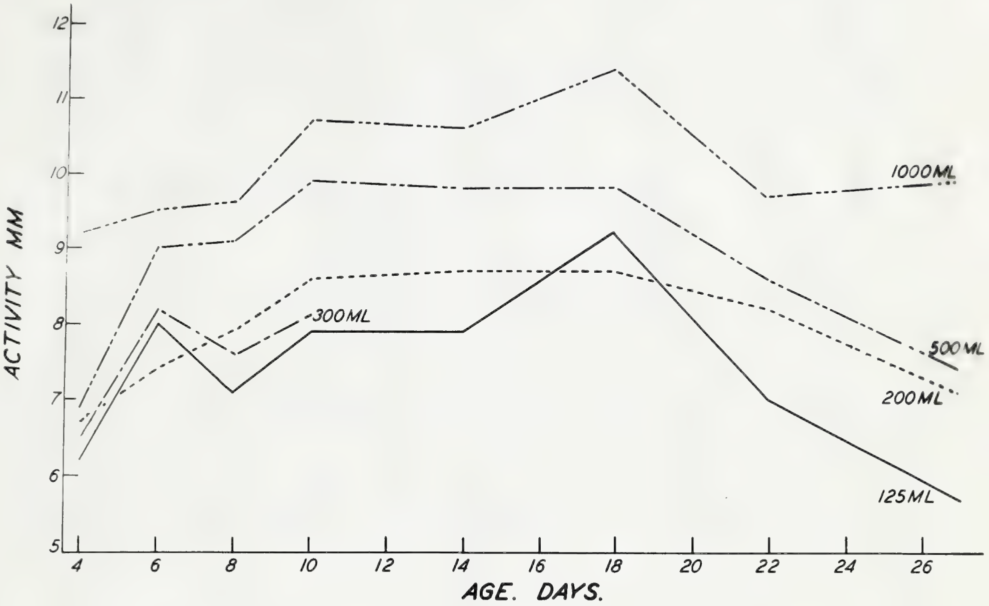

Figure 3

Effect of depth of medium on the activity
of B. subtilis A32 cultures

It appears very evident that the greater the surface area exposed in relation to the volume of medium, the greater is the activity produced in the culture. This may, however, be entirely due to an actually greater amount of growth where there is more area exposed, rather than to the effect of aeration per se. To confirm whether it was an aeration effect, one would have to aerate deep cultures artificially with sterile air, or else employ a constant shaking machine. We were not equipped with this type of apparatus and had to employ stationary cultures. For such cultures at least the shallower the layer of medium the greater is the activity of the culture over a period of many days.

Effect of Temperature and Period of Incubation on the Antibiotic Activity of A32

In the first test, .03% asparagine medium, at the rate of 50 ml. per flask, was added each day to twelve 200-ml. Erlenmeyers and inoculated with A32. Three flasks were incubated at each of 25°, 30°, 35°, and 40°C. This process was carried on for 12 days. On the 12th day, therefore, there were cultures at each incubation temperature from 0 to 12 days old. On the 12th day each culture was tested for

activity. Eight discs were used for each flask. The figures presented in Table VI are averages based on 24 discs (3 flasks x 8 discs).

TABLE VI

Effect of temperature and period of incubation on activity (mm.)

Period of incubation (days)	Temperature			
	25°	30°	35°	40°
2	-	5.1	7.6	3.9
3	4.9	8.2	6.8	3.4
4	6.8	8.3	8.1	-
5	7.3	10.2	6.9	5.7
6	-	10.2	-	7.4
7	-	10.2	-	-
8	9.4	9.5	8.1	9.9
9	10.8	9.3	9.2	-
10	-	-	-	-
11	11.5	9.7	9.3	-
12	13.5	10.5	9.6	-

Results of this test were quite erratic in that many cultures did not "catch" and others that grew showed only little activity. Many that were obviously out of line were not included in the table. Other plates in this test became badly contaminated and were not included. Another error may have arisen through the use of fresh lots of medium made up as the test proceeded. These may have varied somewhat. The results of this preliminary test are pre-

sented, however, because they seem to point to the fact that temperature of incubation is important, especially in the first few days of growth. At a lower temperature it takes longer for a peak activity to be reached, while at a temperature above 35^{o} the peak activity is not as great as at lower temperatures. The results in the individual flasks also showed that as soon as growth began there was activity in the culture. This would tend to indicate that the antibiotic from A32 is elaborated during growth and is not a breakdown product of the cells. This observation seems to be confirmed by another characteristic often noted. This was that when test plates of Xan. 190 accidentally became contaminated with A32 small halos were evident around the colony even if the colony were the size of a pinhead. The results in Table IV are presented to give a picture of the general trend.

A second test was carried out in the following manner: four 2-litre flasks, each containing 500 ml. of .03% asparagine medium, were inoculated with A32. One flask was incubated at each of 25^{o}, 30^{o}, 35^{o}, and 40^{o}C. As soon as growth was evident the cultures were tested for activity every day, using four test plates per flask per day. Readings were taken until the activity reached a low figure for each flask. The results are presented in Table VII and in Figure 4. They are average readings over five-day intervals.

TABLE VII

Effect of temperature and length of incubation period on activity (mm.)

Period of incubation (days)	Temperature 25°	30°	35°	40°
0 - 5	6.6	8.7	9.6	9.3
6 - 10	9.7	11.9	11.0	9.7
11 - 15	10.4	11.5	10.7	7.4
16 - 20	9.0	10.7	8.8	5.1
21 - 25	9.8	9.3	7.5	1.5
26 - 30	8.8	9.3	6.6	
31 - 35	7.7	4.8	3.7	
36 - 40	7.7	2.1	2.0	
41 - 45	7.7			

Combining the results into five-day periods levelled off the slight daily variations due to experimental error. An examination of Table VII shows the peak activity to be reached between the sixth and tenth days at 30°, 35°, and 40°C., whereas at 25°C. the peak is reached somewhat later after which the activity begins to go down, falling much more rapidly at the higher temperatures, the 25°C. series still showing an activity of approximately 7.7 mm. at 45 days. The 25°C. series was kept on after the termination of the test and it still showed an activity of around 7 mm. at 60 days.

One of two factors might cause this inactivation of the culture. The active material being elaborated during the early stages of growth of the culture might be

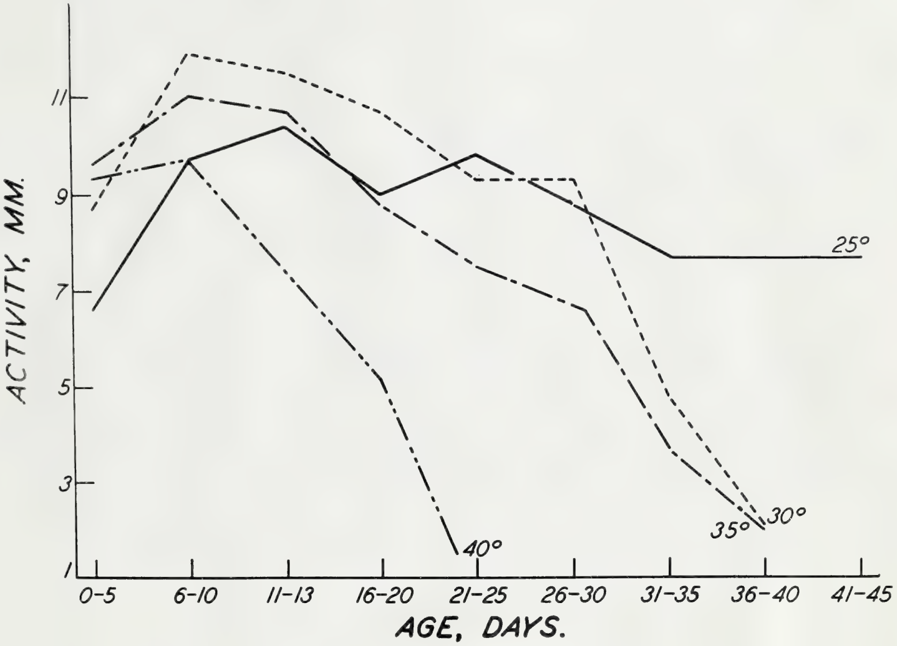

Figure 4

Effect of temperature and length of incubation
period on the activity of B. subtilis
A32 cultures

inactivated by breakdown products of the culture. The other factor might be a loss of activity due to prolonged exposure to higher temperatures, since other tests too show its relative stability at lower temperatures.

To test these hypotheses the following test was run: Two 500-ml. flasks, each containing 100 ml. of .03% asparagine medium, were inoculated with A32 and incubated at 30°C. for eight days. Both cultures were then tested for activity. One of the cultures was then extracted with n-butanol (discussed under Extraction Studies). The butanol extract and the remaining culture were put back at 30°C. and the activity of each was tested at intervals. The results are presented in Table VIII and in Figure 5.

TABLE VIII

Activity (mm.) of A32 culture and butanol extract when kept at 30°C.

Period of incubation (days)	Activity	
	A32 culture	Butanol extract**
8	10*	15.0
13	10	10.9
17	7.7	8.6
21	9.1	8.6
25	8.7	8.4
30	8.9	9.5
35	8.4	9.0
39	8.1	7.5

* Cultures in both flasks showed an activity of 10 mm.

** The butanol extract, though just freshly extracted, was considered to be as old as the culture when the readings were begun.

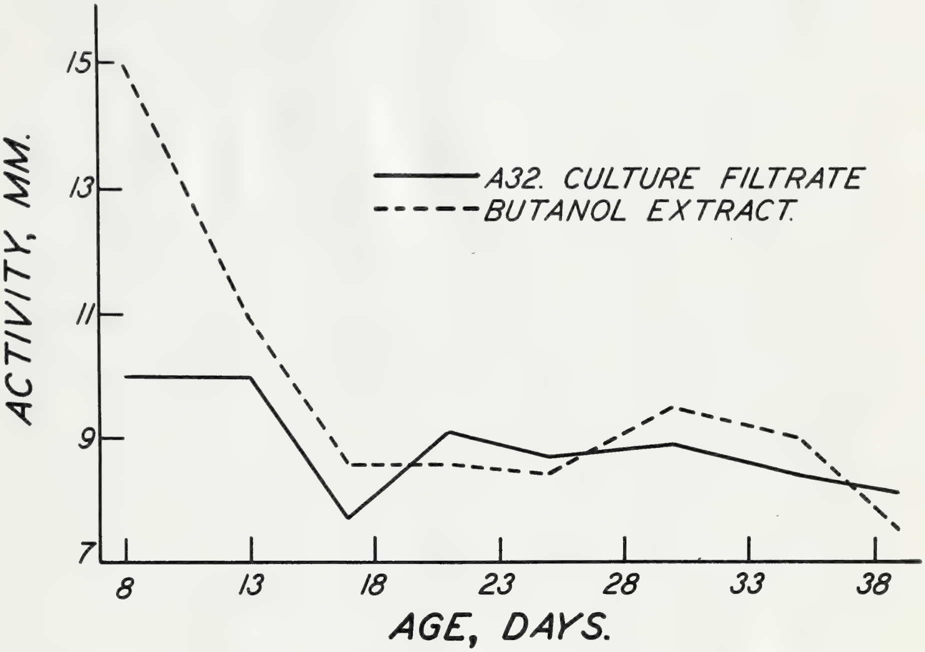

Figure 5

Activity of B. subtilis A32 culture and a butanol extract from it, when kept at 30°C.

In this test it was assumed that the butanol extract would contain few if any breakdown products, whereas the culture would go on accumulating them. Any parallel decrease in the activity of both extract and culture would be due thus to a time-temperature effect. An examination of Table VIII and Figure 5 shows that this was the case. Except for the big drop in the activity of the extract immediately it was placed at 30°C., the culture and extract follow quite a parallel course, with less than 1 mm. difference between them. Since measurements were made only to the closest mm. the differences are not considered significant. The difference of 10 mm. to 15 mm. on the first day might also be interpreted on the basis that the culture at that time was 8 days old, whereas the extract was actually 1 day old, even if it were taken from an 8-day-old culture. It would seem, therefore, that the gradual decrease in activity of the earlier tests is not due to an interaction between the active principle and breakdown products in the culture, but rather to prolonged exposure to elevated temperatures.

STUDIES ON EXTRACTION AND PURIFICATION OF THE ANTIBIOTIC OF A32

Exploratory Methods

Early in the investigation extraction studies were begun. Different methods at one time or another seemed promising but were abandoned when other methods were found. Though much of this exploratory work yielded no satisfactory extraction method, it will be presented since it may prove useful in any future chemical studies.

It was found that Nuchar, an activated charcoal, when mixed in small amounts with staled 5% bran-extract medium for 10 to 15 minutes, completely removed the active principle from the medium. Little or none of the activity could be removed from the Nuchar by any of the following: chloroform, acetone, ether, petroleum ether, benzene, or xylene. Buffered phosphates did remove some of the activity.

Several ether extractions of staled bran-extract medium, using a total of about 10% ether, yielded an active amber-colored solution with some activity. Saturation of the staled media with table grade NaCl was of some benefit in the extraction, but a great deal of activity still remained in the medium. The activity and color of the

ether extract could mostly be removed by a column of 1 to 1 alumina and super-cell. The column released some of the active material onto test agar but it retained a great deal of it even after repeated ether or phosphate buffer extractions.

Acidifying staled bran media with concentrated HCl below its normal pH of approximately 6.5 had no effect on the efficiency of ether extraction. It was noted, however, that a precipitate formed at a pH of 4 to 5. This precipitate proved fairly active and the staled media less active after precipitation than before.

Some work was done on dried acid precipitate. It was found that 5 or 6 extractions of the precipitate with acetone removed most of its activity, while evaporation of the acetone extract yielded a fairly active oily material. Ethyl alcohol (70%) and ether, especially with some alcohol added to it, would also remove some of the active material from the acid precipitate. Several extractions of the finely ground precipitate with small amounts of solvent were found to be more effective than extraction with a larger amount of solvent.

For a time the following method of extraction of staled bran-extract media seemed to show some promise:

1. Precipitate with concentrated HCl and dry the precipitate;
2. Grind the precipitate to a fine powder, extract several

times with small amounts of ether plus 70% ethyl alcohol;

3. Evaporate the extract under a partial vacuum. This yielded a brown, oily liquid which was quite active;

4. Dissolve the liquid in very dilute warm H_2SO_4. Cool and extract several times with ether;

5. Add one or two volumes of petroleum ether to the ether extract. Evaporate.

On evaporation a brown, gummy substance, showing a great deal of activity, remains. Though this method yields an active fraction it falls down in that the original acid precipitation does not completely remove the activity from the staled medium, nor do several ether extractions completely remove the activity from the acid precipitate.

It was found that if tannic acid were used to precipitate staled bran-extract media or media containing corn-steep liquor, a much finer precipitate resulted than when HCl was used. Several extractions with 70% alcohol removed most of the activity from this precipitate. Evaporation of the alcohol extract yielded a largely inactive fraction. If, however, the alcohol extract were evaporated to a small volume at 25° to 30°C., at reduced pressure, a precipitate formed. The filtrate, if cooled in a freezing mixture of ice and NaCl, yielded still more precipitate. Repeated warming and cooling ten times still produced more precipitate. These precipitates were not very heavy but, on bulking and

testing them, they proved to be fairly active, the final filtrate showing only little activity.

Acid precipitation of staled media is the first step in the purification of tyrothricin (6) and subtilin (17), after which the acid precipitates are extracted with alcohol. Upon the addition of 1% NaCl solution to the extracts active precipitates of tyrothricin or subtilin form. An attempt was made to use this method with A32. Upon the addition of the NaCl a slightly active precipitate was formed. The alcohol extract still remained active.

None of the methods attempted in the exploratory work produced a complete extraction. Precipitation by HCl or tannic acid still left active supernatant liquids. Precipitation by tannic acid, though yielding a finer precipitate than HCl, required much more acid to produce complete precipitation, nor was the "end point" as sharp as when HCl was used. In both cases it appears as if it were mainly a precipitation of proteins in the medium which carry some of the active material down with them, rather than a precipitation of the active material as such. Further treatment of such precipitates with solvents still leaves active residues. Ether extractions of staled media, too, do not make a complete job.

Butanol Extraction Method

In the purification of bacitracin (18), decanted staled medium is extracted with n-butanol. A preliminary test on staled 5% bran-extract medium, using butanol, yielded a very active extract. Checks showed pure n-butanol to have no antibacterial effect. The n-butanol extract when evaporated on a watch glass over a water bath, yielded a very active brown material which, when ground and mixed with large amounts of Dixie Clay*, still remained quite active. Further tests using this method were undertaken.

It was found that several butanol extractions removed almost all the activity from staled media. Concentrating staled media to a smaller volume had no beneficial effect on extraction. Preliminary extraction with a small amount of petroleum ether took out none of the active material, but did seem to increase the efficiency of subsequent butanol extraction. Residual activity in the medium was believed to be due to dissolved butanol remaining in it, since butanol is soluble in water to the extent of about 8%. This butanol could be almost entirely removed by salting out with NaCl. This procedure completely removed all traces of activity from staled medium.

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* Obtained through the courtesy of the Naugatuck Chemical Division of the U. S. Rubber Company, Naugatuck, Conn.

The method finally adopted for extraction of staled media was as follows: The culture is filtered through cheesecloth or is decanted to remove the bacterial growth. The liquid is first extracted with a small amount of petroleum ether. Enough n-butanol is then added to the medium so that a layer of it is formed on the top after the medium is shaken. This extract is removed in a separatory funnel. The medium is then extracted two or three more times with n-butanol. A total of about 15% of butanol is used. The medium is then warmed slightly and is saturated with table grade NaCl. This causes most of the residual butanol to separate out. The bulked butanol extracts contain all the active material, the residue being inactive. It is important to allow sufficient time for the successive butanol extracts to separate out. The amber-colored extract obtained by this method is hereinafter referred to as butanol extract.

Use of Various Adsorbents

Studies on various adsorbents were undertaken with two purposes in view. The first was to aid in any further purification studies, since Nuchar and alumina had shown some value in this respect in the early studies. The second was to find some inert carrier or diluent for the active

material with a view to using it as a seed protectant. An early test (discussed under Stability Tests) had shown Dixie Clay to be a good diluent.

The adsorbents were compared for the most part by passing staled medium or butanol extract through a column of each 4 inches long by one-half inch in diameter in an adsorption tube made out of soft glass test tubes. The medium, or the extract, was allowed to percolate through the column but, when this percolation was too slow, the column was attached to a suction flask which was then attached to a water-vacuum pump. The liquid coming through the column--here referred to as filtrate--was tested for activity by the usual paper disc technique. The columns were tested for activity by placing loopfuls of material or streaking some of it across test plates. All the materials tested were by themselves inactive.

In one experiment columns of Dixie Clay, Pyrax ABB*, and alumina were heated at 170°C. for 48 hours. Butanol extract was then put through the columns. The column of Dixie Clay soon became sticky and stopped any movement of the extract, and even when attached to the vacuum pump the extract did not come through very readily. The filtrate had most of the color and activity of the original extract. The

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* Obtained through courtesy of R. T. Vanderbilt Co., Inc., New York, N.Y.

column, when tested, showed some activity, but this is attributable to the fact that it was very wet and still retained some extract on it. Pyrax ABB was as ineffective as was the Dixie Clay. The alumina, on the other hand, allowed the butanol extract to pass through very readily. The color was removed in the first half inch of the column. This colored part, when tested, released a good deal of activity onto the agar, the colorless part being inactive. The filtrate was completely inactive.

A series of experiments on various adsorbents was run using staled .03% asparagine medium. In the first experiment the stale medium was put through originally dry columns twice. In the second, water was allowed to percolate through the columns after which they were sterilized in the autoclave. In the third, all the adsorbents except Nuchar were heated to red heat in a crucible before being used. The results are summarized in Table IX.

The efficiency of alumina and Nuchar as adsorbents had been indicated in the early extraction studies. This was confirmed by the foregoing tests. Preliminary tests had also shown soil to be a good adsorbent and this too was confirmed, both sterile and unsterile soil being effective in this respect. The efficiency of soil seems to be a property of the fine particles in it, rather than of the organic matter. This is shown by the fact that mixing black soil with sand

TABLE IX

Comparative effectiveness of different adsorbents in removing the antibiotic of A32

	Activity* of filtrate (mm.)		
	Experiment number		
Adsorbent	1	2	3
Original staled medium	9	9	9
Pyrax ABB	0	6-7	4-5
Dixie Clay	3	-**	0***
Nuchar	0	2-3	0
Infusorial Earth #0	8-9	8-9	5-6
Talc	2	5-6	0***
Super-cell	9	8-9	6-7
Alumina	-	1	0
Unsterile Edmonton black soil	2	0	0
Sterilized Edmonton black soil	-	0	-
Edmonton soil:Sand (1:1)	-	7-8	-
Edmonton soil:Sand (1:2)	-	6-7	-

* Readings of activity are based on 8 discs
** Dash indicates that that adsorbent was not tested
*** Had to be sucked through with the vacuum pump.

reduced the efficiency greatly and also by the fact that heating unsterile soil to red heat (Experiment 3), thus burning out the organic matter, did not reduce its efficiency. Dixie Clay and talc, if first heated to red heat, will remove the activity from staled media; they have the serious disadvantage, however, that they become quite sticky and that liquids pass through only with difficulty. Pyrax ABB, Super-cell, and Infusorial Earth #0, do not from these experiments show much promise as adsorbents. Materials such as Dixie Clay, Pyrax

ABB, and talc, might, however, be quite effective as inert carriers in a mechanical mixture, Dixie Clay at least having shown some promise as described under Stability Tests.

A test was undertaken to determine the effectiveness of different types of soil in removing the active material from staled .03% asparagine medium. Two series were run: (1) the soil columns were sterilized in the autoclave and were then allowed to dry thoroughly until they were quite porous before staled medium was put through; (2) the staled medium was put through the columns when they were still damp and fairly compact after autoclaving. A different lot of staled medium was used for each series. The results are presented in Table X.

TABLE X

Comparative effectiveness of different soils in removing the antibiotic of A32

Soil	Activity* of filtrate	
	Dry series	Moist series
Original staled medium	9.7	7.7
Sand	6.0	3
Brown prairie loam	5.5	trace
Gray wooded	5.8	0
Sandy loam	7.0	trace
Edmonton black soil	0	0
Edmonton black soil:Sand (1:1)	-	3

* Readings of activity are based on 8 discs.

It was noted that the staled medium went through the dry porous series very readily, with the exception of the Edmonton black soil through which it percolated slowly. It will be noted too that in the dry series the Edmonton black soil was the only one completely removing the activity from the staled medium. The medium percolated through all the soils in the moist series much more slowly, and it can be seen that the soils in this series are much more efficient. Any soil with a fine fraction in it seems to be effective; the higher the proportion of sand in it the less effective it is. The efficiency depends a great deal on how quickly the medium percolates through. Clay was also tested but it soon became sticky and let through very little medium in either series. The results for it are therefore not included in Table X.

Further Purification Studies

Since Nuchar and alumina absorbed the activity from butanol extract, attempts were made to elute the active material from them in the hope of getting a purer fraction. Ethyl alcohol, both 70 and 90%, acidified or non-acidified were ineffective in removing any of the activity even after allowing the extraction to proceed for 24 hours. Ether removed some activity. The ether eluate, when evaporated,

yielded a thin layer of fairly active gummy material. Phosphate buffers at pH's of 5.3, 6.2, 7.1, and 8.0, though removing some of the active material left a great deal of it on the alumina. Ordinary water was as effective as were the buffers. Sodium hydroxide, .01 M. took out very little active material.

Nuchar retains the active material very tenaciously. At no time was it ever observed to release any onto test plates. Alumina, though it retains the active material after repeated extractions, will still release enough onto the test agar to produce sterile zones.

Other preliminary attempts to remove the active substance from butanol extract were also made. Water extraction was found ineffective. In the extraction of bacillin (18) the butanol extract is concentrated by steam distillation in vacuo. A test using steam distillation on the butanol extract from A32 staled media was inconclusive though this method may have some possibilities.

Dialysis through collodion membranes may offer some possibilities as a purification method. When butanol extract was dialysed against pure n-butanol, material which was both colored and active came through the collodion. A similar test dialysing staled .03% asparagine medium against water gave similar results.

Butanol extract is the most active fraction thus far obtained. As was mentioned earlier, this extract can be ures at pH's of 5.7, 6.0, 7.1, and 7.4, though

one of the active material [illegible]

ruins. Ordinary yeast [illegible]

Coffee cylinders, 0.1 N, [illegible]

[illegible]

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Other preliminary attempts to [illegible]

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found ineffective. In the [illegible]

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ies as a purification method. [illegible]

ed against [illegible]-butanol, [illegible]

active [illegible]

ding, obtained. [illegible]

results.

evaporated to dryness over a water bath, after which it can be scraped off to give a good yield of an active brown powder. Tests show it to contain both nitrogen and sulphur* though there has been no consistency in the amounts between two different lots of powder prepared in this way.

At this point some of the differences between the antibiotic from A32 and those isolated from other B. subtilis strains, as far as extraction methods are concerned, can be assessed. The antibiotic from A32 is found, as are bacillin (7) and bacitracin (18), mostly in the cell-free filtrate of staled media. In this respect they are quite different from subtilin (17), which is found predominantly in the cells of the subtilin-producing strain.

The method used for the production of subtilin--i.e. a precipitation of staled media with acid and subsequent alcohol extraction of the precipitate--has not been found very effective in the case of the antibiotic from A32. Jansen and Hirschmann (17) suggest that subtilin activity is probably due to more than one substance and this may be the case also with A32.

Foster and Woodruff (7) find treatment with 2% Norite to remove all activity from their staled broth. This is similar to our results with Nuchar. They find, however, that bacillin can be eluted from the Norite by ethanol. At

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* I am indebted to Mr. C. O. Gerbrandt for running the tests on nitrogen and sulphur.

no time have we been able to remove any of the active material from Nuchar. It may be, however, that the two charcoals are different and lend themselves to different techniques.

Though bacitracin is extracted from staled medium with n-butanol, it seems to differ from the active material of A32. However, the data available on bacitracin are rather meagre.

ANTIBACTERIAL STUDIES

The field of associative and antagonistic relationships of microorganisms has been reviewed by Waksman (34). Plant Pathologists have long recognized the occurrence, but more recently, the importance of antagonisms between organisms and especially between soil microorganisms and plant pathogens. Garrard and Lochhead (8) have reviewed this aspect of the field. More recently Ark and Hunt (1) reported Bacillus vulgatus and an unidentified yellow bacillus to be active against a number of phytopathogenic bacteria and fungi as well as against other microorganisms.

With the isolation of specific antibiotics there have appeared reports on the in vitro action of some of these against phytopathogens (3, 9, 28, 29, 37, 38). No reactions of plant pathogens to the antibiotics thus far isolated from B. subtilis (7, 17, 18) have as yet been reported. That such

might offer a fruitful field of study, however, is evident from other recent work. Katznelson (19) found *B. subtilis* as well as other spp. of *Bacillus* to produce a thermostable substance toxic to *Rhizoctonia solani*. Humfeld and Feustel (16) tested sterilized staled media of *B. subtilis* (the strain subsequently used in the isolation of subtilin (17)) against a number of bacteria including the three phytopathogens, *Erwinia amylovora*, *Xanthomonas juglandis*, and *Phytomonas michiganensis*. They found *P. michiganensis* to be inhibited at very high dilutions of the staled medium, while the other two, both gram negative, were not affected.

The antibacterial spectrum of an antibiotic is often characteristic enough to identify it. For strict comparisons between antibiotics similar conditions of testing should be employed. However, a spectrum may sometimes be distinct enough to identify an antibiotic under different conditions. With a view to obtaining a spectrum for the antibiotic from A32 and of finding seed-borne pathogens against which it might be effective, antibacterial tests were carried out at different times in the investigation. A list of the organisms tested is given in Table XI. Several antagonistic actinomycetes, as well as a number of the bacteria originally isolated, were also tested.

TABLE XI

Organisms employed in antibacterial tests

Name of organism*	Special designation	Source
Xanthomonas translucens f.sp. hordei-avenae	Xan. 190	Dr. G. Semeniuk, Ames, Iowa
f.sp. cerealis	Xan. 1015	Dr.W.A.F.Hagborg, Wpg.
f.sp. hordei-avenae	Xan. 1016	" " "
f.sp. hordei	Xan. 1017	" " "
f.sp. undulosa	Xan. 1018	" " "
Pseudomonas coronafaciens	3003	" " "
Bacillus brevis		Dr.A.G.Lochhead, Ottawa
" cereus		" " "
" vulgatus		" " "
" mycoides		" " "
" megatherium		" " "
Sarcinia lutea		" " "
Escherichia coli	communis	Dr. A.M. Shaw, U. of A.
" "	communior	" " "
Pseudomonas pyocyaneus		" " "
Serratia marcescens		" " "
Staphylococcus aureus		" " "
Bacillus mesentericus		" " "
" subtilis	30	" " "
" "	33	" " "
" "	16	Dept.of Plant Sci. U.of A.
Erwinia amylovora**		" " " " "
Corynebacterium sepedonicum		" " " " "
Aerobacter aerogenes		" " " " "
Pseudomonas atrofaciens**		" " " " "
Erwinia carotovora		Dr. J. G. Leach, W.Va.
Agrobacterium tumefaciens		Dr. A.J. Riker, U. of Wisc
Aerobacillus polymyxa	7070	Amer.Type Culture 7070
Pseudomonas Woodsii		Dr.M.W.Cormack, Edmonton
Corynebacterium insidiosum		" " " "
Bacterium agropyri**		" " " "

* Names of phytopathogens are according to Weiss and Wood (40)

** Identity has not been confirmed.

Preliminary Tests

During the isolation of the antagonists, *Xan. 190* and *Pseudomonas coronafaciens* were found to be susceptible to the inhibitory action of A32 as previously described. *Aerobacillus polymyxa* 7070 was also found to be very susceptible in these tests.

During the course of exploratory work on extraction methods, antibacterial tests were carried out with some of the active fractions. In one such test loopfuls of tannic acid precipitate were spot-inoculated on test plates of a number of bacteria prepared in the manner used with *Xan. 190*. The halos around the spots of precipitate were taken as a measure of antibacterial activity. One series in this test employed nutrient agar, the other series potato-dextrose agar, in the test plates. The results are presented in Table XII.

The results of Table XII show that the precipitate is active against a number of pathogens. It had been noted during the early work that the inhibitory effect of A32 against *A. polymyxa* was much greater when potato-dextrose agar was used than when nutrient agar was employed. This was confirmed in the foregoing test and appears to hold for some of the other organisms as well. Nutrient agar was, however, retained as the test agar for the following tests, since it

TABLE XII

Activity (mm.) of tannic acid precipitate against several species of bacteria

Test organism	Nutrient agar	Potato-dextrose agar
Xan. 190	7	-
B. subtilis 16	0	0
Cor. sepedonicum	-	5
A. aerogenes	2	0
Ps. atrofaciens	4	9
E. carotovora	2	1
Ag. tumefaciens	5	5
A. polymyxa	3	7

was well adapted to Xan. 190 and it was desirable at this stage to keep the results comparable for all the test organisms.

In a second experiment test plates of a number of organisms were prepared. These plates were then spot-inoculated with A32 and incubated at 30°C. The results are presented in Table XIII.

Most of the organisms in the foregoing test were sensitive to a greater or lesser degree. Others might perhaps have shown greater sensitivity had A32 been allowed to make some growth and the organisms then been applied by spraying them on the plates. In comparison with Xan. 190, which is very fast growing, the slowness of the other strains of X. translucens is notable.

TABLE XIII

Activity of A32 on spot-inoculation

Test organism	Notes on halos	
	Hours required to appear in plates	Width in mm.
Xan. 190	15	6
Xan. 1015	72	9
Xan. 1016	72	14
Xan. 1017	72	9
Xan. 1018	72	9
Ps. coronafaciens	15	6
S. lutea	15	5
E. coli communis	15	trace
E. coli communior	15	0
Ps. pyocyaneus	15	trace
S. marcescens	15	3
Staph. aureus	15	3 (diffuse)
B. subtilis 16	15	7
E. amylovora	36	9
Cor. sepedonicum	72	trace
A. aerogenes	15	4
Ps. atrofaciens	72	11
E. carotovora	72	trace
Agro. tumefaciens	15	4
A. polymyxa	Very little growth	

Tests Employing Butanol Extract

When the butanol extraction method was adopted a preliminary antibacterial test against a number of organisms was carried out. Butanol extract was applied to the test plates using filter paper discs. Of all the organisms tested Xan. 190 proved to be the most susceptible, showing 15 to 17

mm. wide halos. E. amylovora, Ps. coronafaciens, Ag. tumefaciens, E. carotovora, and Ps. atrofaciens were also quite susceptible. S. lutea, A. aerogenes, and the E. coli strains seemed to show some sensitivity. All other organisms tested showed only doubtful halos. Hagborg's strains of X. translucens appeared sensitive but their slowness in appearing made the results inconclusive.

A more extensive series of tests, using a butanol extract of staled .03% asparagine medium was undertaken. It was decided to test first the relative susceptibility of the different X. translucens strains. These results are given in Table XIV.

TABLE XIV

Sensitivity of strains of X. translucens to a butanol extract of A32

Strain of X. translucens	Time before measurements could be made (hours)	Width* of halo (mm.)
Xan. 190	18	13
Xan. 1015	72	16
Xan. 1016	96	25 in one plate; complete inhibition in the other
Xan. 1017	96	Complete inhibition
Xan. 1018	72	17

* Average of 6 discs for each strain.

It is very apparent that all of the *X. translucens* strains are very susceptible to a butanol extract of A32.

It was desirable to obtain faster plate results with Hagborg's *X. translucens* strains. In order to do this 1 ml. of a 4-day-old broth culture was used to seed the plates instead of the usual water suspension. A number of the other organisms were also tested in a similar manner. The butanol extract used was the same one as in the foregoing experiment. The results, listing the different organisms in the order of their sensitivity, are presented in Table XV. The values given are average ones based on measurements from six separate discs. Variations between individual discs in this test was quite small.

TABLE XV

Activity of butanol extract of A32 cultures using 1 ml. of broth culture of the test organism per plate

Test organism	Width of halo (mm.)	Test organism	Width of halo (mm.)
Xan. 1016*	12.3	S. lutea	3.3
Xan. 1017*	11.8	Ps. pyocyaneus	3.2
Xan. 190	10.7	E. coli communis	1.8
Xan. 1018*	10.2	E. coli communior	1.5
B. megatherium	9.8	B. mycoides	1.3
A. tumefaciens	9.3	B. subtilis 30	1.3
Xan. 1015*	8.7	B. subtilis 16	1.3
E. amylovora	7.7	B. vulgatus	1.0
Ps. atrofaciens	7.0	S. marcescens	1.0
Ps. coronafaciens	5.3	B. cereus	1.0
B. brevis	5.2	A. aerogenes	trace
B. mesentericus	4.8	A. polymyxa	trace
E. carotovora	4.8	B. subtilis 33	trace
Cor. sepedonicum	4.5	Staph. aureus	trace

* Did not appear until after 4 days

In the foregoing test a number of the antagonistic bacteria isolated at the beginning of the investigation were also tested. Ten out of 13 tested proved susceptible to butanol extract, the values obtained ranging from 5.8 to 1.3 mm. Two actinomycetes, A56 and A62, were also susceptible, exhibiting 4.5 and 5.0 mm. halos respectively. Using a broth culture instead of a water suspension to seed test plates did not induce Hagborg's strains of _X. translucens_ to appear any more quickly. It was noted, however, that the halos when using a broth suspension were much smaller than when a water suspension was used. A comparison of Table XIV and Table XV makes this clear. Further discussion of this question will be found in a subsequent section under the heading, Factors Affecting Halo Width.

Tests Comparing 1:1000 Subtilin and Butanol Extracts from Cultures of A32 and B. subtilis #231

A sample of subtilin was kindly supplied by the Western Regional Research Laboratory, U. S. Department of Agriculture, at the request of the National Research Council. A culture of _B. subtilis_ #231 (of N.R. Smith), used in the production of subtilin by Jansen and Hirschmann (17), was also supplied by the National Research Council. A comparison

of A32 and B. subtilis #231 is given in a subsequent part of the thesis.

Tests were carried out to compare the antibacterial spectrum of subtilin with that of a butanol extract of A32 cultures and with that of a butanol extract of B. subtilis #231 cultures. The extracts were prepared by growing cultures of A32 and #231 under similar conditions in .3% asparagine medium (this medium being optimum for subtilin production (17)). The staled media were then extracted with equal amounts of n-butanol. The subtilin employed was a 1:1000 dilution in sterile distilled water.

Two such tests were carried out. In the first 10 ml. of agar were used per test plate. The second employed 15 ml. of agar, since it was believed a thicker layer of agar would remove some of the variability in halo width which often occurs when Petri plates with irregular bottoms are used. Some of the organisms that appear to show a differential reaction are presented in Table XVI. E. carotovora, A. tumefaciens, Ps. Woodsii, and Actinomyces sp. A62 are notable in this respect. The other organisms tested did not behave consistently for some reason as yet unexplainable.

From the figures in Table XVI it can be seen that 1:1000 subtilin produces much smaller halos than does the butanol extract from A32. In the foregoing test only the figures for the two butanol extracts are strictly comparable. Since the concentration of antibiotic in them is unknown, they

TABLE XVI

Antibacterial activity (mm.) of butanol extract of A32 and #231 staled media and 1:1000 subtilin

Test organism	1:1000 subtilin	Butanol extract of cultures of	
		A32	#231
Xan. 190	5	13	5
Xan. 1015	6	10	1
Xan. 1016	9	13	2
Xan. 1017	7	11	2
Xan. 1018	3	11	2
Ps. coronafaciens	1	4	1
B. megatherium	3	8	3
E. amylovora	4	9	2
Ps. atrofaciens	2	8	2
E. carotovora	0	5	0
A. tumefaciens	0	8	0
A. polymyxa*	9	20	13
Ps. Woodsii	0	8	2
Actinomyces sp. A62	0	6	1

* Tested using P.D.A. All others tested on N.A.

cannot be directly compared with subtilin. It is likely, however, that the antibiotic in the butanol extracts was much less dilute than 1:1000. The butanol extracts are, moreover, in all probability not pure, whereas the subtilin is. The lower activity of subtilin as compared with A32 butanol extract is hardly attributable to low diffusibility since, as will be seen from Table XVI, in two cases at least (A. polymyxa and Xan. 1016) halos of 9 mm. width were formed in the subtilin plates. It may easily be, however, that the test conditions were unfavorable to subtilin and that the

use of potato-dextrose agar or the use of a serial dilution method would have given different results. Under the conditions of the test, the substance from A32 has a different spectrum than has subtilin. Against the other organisms tested and not reported in Table XVI the two antibiotics reacted similarly. It will be noted that the butanol extract of B. subtilis #231 cultures also showed some activity, but that on the whole it was much lower than the extract from A32. B. subtilis #231 is a subtilin-producing strain but it may be that a high titre of subtilin was not produced since trace elements were not provided in the medium. Trace elements are known to increase subtilin production (17). Moreover, had a high titre of subtilin been produced in the #231 cultures it is not known whether or not butanol would have extracted it.

Salle and Jann (30) tested the action of subtilin of various organisms. Some of the species were also used in our tests. They employed the paper-disc and agar-cup techniques. At a dilution of 1:1000 they found B. cereus, S. lutea, and Staph. aureus to be very susceptible and they also found B. megatherium to be susceptible. In our tests we also found these organisms to be susceptible to a greater or lesser extent. They found A. aerogenes, E. coli, and S. marcescens to be resistant. This is similar to our results. Salle and Jann (30) used 0.1 ml. of a broth culture to seed 20 ml. of agar, but the type of agar medium used is not

stated. The results are thus not strictly comparable, but tend to be confirmatory as far as they go.

Bacteriostatic Action of A32 and Subtilin on Xan. 190

In no case during the course of the work were colonies of Xan. 190 found to invade the sterile halos, regardless of the length of time the plates were kept. This was true whether staled A32 medium or butanol extract was used. Except for one isolated instance with A. polymyxa none of the other test organisms was found to invade these areas. Transfer of pieces of agar from sterile areas of the Xan. 190 to fresh agar did not yield Xan. 190. From such evidence it may be deduced that the antibiotic produced by A32 is bactericidal as well as bacteriostatic in its action.against X. translucens. From similar evidence Rudolf (29) concluded that penicillin is bactericidal in its action against Xanthomonas juglandis and Erwinia amylovora.

That such is not actually the case with Xan. 190 and A32 can be seen from the following. Bits of agar were removed from the sterile zone and from the area immediately under the paper disc, and incubated in nutrient broth at 30°C. A parallel series was run with agar from 1:1000

subtilin plates. After a period of incubation loopfuls of the resultant broth cultures were streaked on agar slants. In all cases typical Xan. 190 was recovered. The antibiotic produced by A32 as well as subtilin are thus only bacteriostatic in their action under the conditions used. The failure to recolonize sterile areas or obtain Xan. 190 when transferred to fresh agar may be due to the tenacity with which the antibiotic substance is retained. As soon as the agar is transferred to nutrient broth it may, on disintegration, so dilute the antibiotic substance that it is no longer effective.

FACTORS AFFECTING WIDTH OF HALO PRODUCED

The diffusibility of an antibiotic substance will influence the size of halo produced. This diffusibility is a function of the molecular weight of the substance. The halo will also vary with the consistency and type of agar used in the test plate. As has been already noted, using nutrient broth cultures instead of water suspensions of Hagborg's strains of X. translucens did not decrease the time required for the halo to appear, but it did reduce the width of halo obtained, as can be seen by comparing Tables XIV and XV.

Effect of Type of "Seed" Suspension on Halo Width

A more extensive test comparing the two methods of seeding test plates was undertaken. In one series 1 ml. of a 48-hour nutrient broth culture of *Xan. 190* was used to prepare the plates, while in the second case 1 ml. of a water suspension was used. Discs bearing butanol extract were applied to the plates, which were incubated at four different temperatures. The complete results are presented in Table XVII.

TABLE XVII

Effect of different methods of seeding test plates on width of halos (mm.) produced

Temperature	1 ml. of H_2O suspension			1 ml. of broth culture		
25°	13	14	14	11	11	11
	14	13	14	10	10	9
	14	14	14	10	10	10
30°	13	14	14	10	9	9
	13	13	13	10	10	10
	14	13	13	10	10	10
35°	15	13	14	10	10	10
	14	13	12	10	9	10
	14	15	14	10	10	10
40°	15	13	14	10	10	10
	15	15	13	10	10	11
	15	15	14	10	10	15

It is apparent from Table XVII that the water suspension gave consistently wider halos. The 15-mm. halo in the 40°C. broth series is probably accounted for by the fact that the agar in that spot was thin because a round-bottomed Petri plate was used. Hayes (13) and deBeer and Sherwood (2) refer to the same effect of thin agar. Each antibiotic appears to have its own "diffusion potential". It is believed now that many individual inconsistencies due to uneven depth might have tended to disappear had 15 or 20 ml. instead of 10 ml. been used throughout the investigation. The results in Table XVII also indicate that temperature of incubation does not have much effect on halo width.

Effect of Time of Application of the Antibiotic on Halo Width

During the course of the work it was desirable to know whether butanol extract had any effect on Xan. 190 in plates where the organisms had already made some visible growth. Two sets of test plates were made up. In the first the discs of extract were applied immediately the agar had sufficiently hardened, as was done throughout the whole investigation. The other set had the discs of extract applied nine hours later when growth was already visible, both sets being incubated at 30°C. throughout the whole time

of the test. After 18 hours readings were taken. The set which had the discs applied immediately had sterile halos 15 mm. wide. Those in the second set were only 5 mm. wide, and _Xan._ _190_ was present in the halos to the same extent as on the whole plate when the discs were applied--that is, there was no lytic effect of the extract on _Xan._ _190_.

Since a great difference in halo width between the two sets was obtained, a more extensive test was set up, Plates of _Xan._ _190_ were made up and incubated at 30°C. Discs of butanol extract were applied at two-hour intervals to a set of two plates, over a 16-hour period. Readings were made 24 hours after the plates were first prepared. The results are presented in Table XVIII.

A test similar to the one reported in Table XVIII, using _Xan._ _190_ and 1:1000 subtilin, gave similar results. These are presented below:

Subtilin applied after hours	Halo width (mm.)							
0	6	6	6	6	7	6	6	6
2	6	5	6	6	5	5	6	6
4	5	5	5	5	5	5	5	5
6	4	4	4	5	4	4	4	4
8*	4	4	4	4	4	4	3	4
10	3	3	3	3	3	3	3	3
12	2	2	2	2	3	2	2	2

* Growth first visible in the plates

The results show very strikingly the reduction in halo width with an increase in the time elapsing between

TABLE XVIII

Effect of time of application of butanol extract on halo width

Time after preparation of plates (hours)	State of growth	Halo width	Type of halo
0	not visible	11,12,13,12,13,13,13,13	Sharp and sterile
2	"	12,11,12,12,11,11,11,11	" " "
4	"	10, 9, 9, 9, 9, 9, 9, 9	" " "
6	faintly visible	8, 7, 8, 6, 8, 7, 8, 8	Slightly ragged and some growth in them
8	visible	5, 4, 5, 5, 5, 4, 5, 5	"Halos have Xan. 190 in them
10	"	3, 3, 3, 3, 4, 3, 3, 3	"
12	"	1, 1, 1, 2, 2, 2, 2, 2	Very ragged
14	"	1, 1, 1, 2, 2, 1, 1, 1	" "
16	"	0, 0, 0, 0, 0, 0, 0, 0	No trace of a halo*

* When the discs were removed after the reading was made, no difference was found in the amount of Xan. 190 between the areas immediately under the disc and those surrounding it.

the preparation of the plate and the application of the antibiotic. Several factors might possibly bring about this effect. As the plates are incubated and Xan. 190 becomes visible on them, there is an increase in the number

of organisms in the plate. This acts as an increase in the amount of "seed" which may affect halo width. The agar may become more firm and rigid as time elapses and this would affect the diffusibility of the antibiotic and thus reduce halo width. As the test organism makes some growth, it may become more resistant to the antibiotic. This would tend to be confirmed by the results on the type of "seed" suspension test (Table XVII) where halo widths, when an actively growing broth culture was used, were less than when a water suspension was used. Various tests were set up to determine whether these hypotheses would explain the results in Table XVIII. These are given below.

Effect of Number of Test Organisms per Plate on Halo Width

In a preliminary test suspensions of Xan. 190 using a standard loopful of the organism in each of 5, 10, 15, and 20 ml. of water were made up. One ml. of the suspensions was then used to seed test plates. Discs of butanol extract were applied and the plates incubated at 30°C. The following results were obtained:

Seed from suspension in	Width of halo in mm.							
5 ml.	11	11	11	11	11	11	11	11
10 ml.	11	11	11	11	10	11	10	11
15 ml.	12	12	12	12	11	11	11	11
20 ml.	13	13	12	13	11	11	12	12

There seems to be an increase in the width of halo as the number of seed organisms is decreased. This increase, however, does not seem great enough to account for the results of the time application test (Table XVIII).

A second experiment was carried out. A suspension of Xan. 190 in sterile distilled water was dispensed into sterile water blanks to give a series of standard dilutions reduced by one-half at each step. One ml. of each dilution was then used to seed test plates, which then had discs of butanol extract applied to them. The results are presented in Table XIX.

TABLE XIX

Effect of amount of "seed" on halo width

Dilution	Width of halo in mm.							
Original	12	12	15	13	12	12	12	12
½ original	12	12	12	13	13	13	12	12
¼ original	12	13	13	13	14	14	14	15
1/8 original	14	14	13	14	14	14	14	13
1/16 original	14	13	14	14	14	12	14	14

These results confirm those of the preliminary experiment in that there is an increase in halo width with a decrease in the amount of seed; however, the amount of

seed does not seem to be the factor in the results of the time of application test (Table XVIII).

Effect of "Age" of Agar on Halo Width

Experiments were carried out to see whether the age--and thus perhaps the firmness--of the agar at the time of application of the antibiotic might have an effect on halo width. In one experiment agar plates were prepared using 10 ml. of agar but without any Xan. 190. As soon as the agar had hardened sufficiently, ½ ml. of a suspension of Xan. 190 made from a 48-hour P.D.A. slant kept at 6° to 7°C. was poured over two of the plates to which discs of butanol extract were then applied. All the plates in the test were then incubated at 30°C. At intervals of two hours two plates were removed, a Xan. 190 suspension poured over the surface, discs of butanol extract applied, and the plates replaced at 30°C. The test was run over a 12-hour period. The following results were obtained: The halos were not as sharp or as regular as those obtained when Xan. 190 is sown in the agar. This is not surprising since there was a slight film of moisture due to the suspension of Xan. 190 on the plates when the discs were applied, and the extract would thus tend to run. The halos, however, did show up well enough to indicate very little difference in their width over the 12-

hour period; they were all fairly close to 12 mm. in width. Checks in which a *Xan. 190* suspension only was applied at the 0- and 12-hour periods showed definite growth over the whole surface of the plates.

A second test representing a variation of the first was next made. Test plates of *Xan. 190* were made up. Discs of butanol extract were applied to two plates at intervals of two hours over a 12-hour period. After 12 hours all the plates had a suspension of *Xan. 190* applied to them by means of an atomizer. All the plates were incubated at 30°C. during the course of the test. Data were taken 48 hours after the plates were first prepared.

The results of this experiment confirm those of the previous one. The halos in this test did not appear until 48 hours after the preparation of the plates and, as in the previous test, they were rather ragged. They were, however, around 15 mm. in width in all cases. Since the only variable in this case was the age of the agar when the discs were applied, this factor does not appear to affect halo width.

In a third test plates of *Xan. 190* were prepared. One set had discs of butanol extract applied immediately. These plates were then incubated at 30°C. The rest of the plates were stored in a refrigerator at 6° to 7°C. After 48 hours the second set was removed, discs of extract were applied, and the plates were incubated at 30°C. The third

set was similarly treated 24 hours later. The results showed that there was no reduction in halo width in the second over the first set of plates and only a very slight reduction in the third set.

None of the experiments on "age", and thus, presumably, firmness of the agar, showed this factor to have much of an effect on width of halos. Any slight differences were far from adequate to explain the results of the time of application test (Table XVIII).

Effect of age of Xan. 190 used to Prepare the "Seed", on Halo Width

In another experiment slants of Xan. 190 of different ages were used to prepare the test plates in order to see whether this might be a factor in halo width. A standard loopful of culture in 5 ml. of water was used to make up the suspension in each case. Discs of butanol extract were applied and the plates incubated in the usual way. The following results were obtained:

Age of inoculum (hours)	Width of halos (mm.)							
12	11	11	11	11	11	12	11	11
18	11	11	11	10	10	10	10	10
24	10	10	10	10	11	10	11	11
48	12	12	13	12	13	12	12	12
72	12	12	12	12	12	12	12	12

The results show that age of inoculum cannot be responsible for the results in Table XVIII. In fact, this preliminary test would tend to show wider halos with older inoculum, though the test was not extensive enough to be conclusive in this regard.

Discussion

Many factors may influence the width of halo produced in the paper-disc or agar-cup method of assay. Several investigators (2, 13, 23) find definitely greater inhibition zones with thin agar, and this has been confirmed in our experience. Miller and Rose (23) and deBeer and Sherwood (2) find the number of test organisms per plate to be a factor, heavier seeding tending to overcome the action of the antibiotic. This tends to be the case also with Xan. 190 and the antibiotic from A32 (Table XIX). Miller and Rose (23) found the length of time elapsing between the preparation of the plate and the application of the antibiotic to be a factor. This is very much the case with Xan. 190 and the substance from A32 (Table XVIII) and subtilin. The reason for this reduction with the lapse of time remains obscure. It does not appear to be due to the number of test organisms per plate or to their age, nor does it seem due to a stiffening of the agar in the plate. It would seem, there-

[illegible]

ble for the results in Table VII. [illegible], this

ny test would tend to [illegible] wider below with [illegible]

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Discussion

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it does not appear to be due to the [illegible] fore, that Xan. 190 becomes more resistant as it makes some growth in the plate.

Miller and Rose (23) also found the protein content of the agar medium to be a factor. This might possibly account for the difference found when broth or water suspensions of Xan. 190 were used to seed test plates, since as 1 ml. of liquid is added to 10 ml. of agar in both cases, the agar concentration would not be a factor.

STABILITY OF THE ANTIBIOTIC FROM A32

Different antibiotics, even when produced by the same species, may vary in their stability. During the course of the work, a number of observations were made and tests were carried out at different times to assess the stability of the antibiotic from A32.

In the early part of the work, staled media were always sterilized by autoclaving at 15 lb. pressure for 15 to 20 minutes before assaying. It was observed that there was usually quite a reduction in activity as measured by halo width produced by the sterilized as compared with the unsterilized staled medium: a staled bran-extract medium was reduced from 10 mm. to 6 mm.; a staled Czapek-Dox medium was reduced from 10 mm. to 4 mm.; a bit of butanol

extract powder in a test tube was reduced from 14 mm. to 10 mm. on autoclaving. Observations had shown no advantage in adjusting the pH before sterilization. The general observation was that, though the substance was not completely thermolabile, there was a considerable reduction in activity on autoclaving. It may be that several substances, only some of which are thermolabile, are produced by A32. Later assays were almost invariably made on unsterile staled media from which the liquid had been decanted or the pellicle removed by filtering through several layers of cheesecloth. It was found that, in the short time required for halos to become visible, there was not enough contamination by A32 to affect the reading. When a .03% asparagine medium was adopted a more extensive series of stability tests was undertaken.

Thermostability of the Antibiotic

Portions of staled .03% asparagine medium were autoclaved for different lengths of time at 113 to 115°C. The results are presented in Table XX. The figures are averages, based on 8 discs.

There is a gradual reduction in activity the longer the staled medium is autoclaved, until after one hour at 113 to 115°C. it is completely inactive.

TABLE XX

Effect of exposure to 113 - 115°C. on the activity of staled medium of A32

	Period of autoclaving in minutes					
	0	10	15	20	25	60
Activity (mm.)	10	7.5	6.0	4.5	4.5	0

General observations tend to show that the antibiotic is fairly resistant to temperatures below 100°C. Evaporation of butanol extract over a water-bath often takes one-half hour, yet the butanol powder obtained is quite active. There was also, during the work, occasion to reduce the volume of staled medium in a boiling water bath for a period of several hours and, though there was some reduction in activity, it was not nearly as great as that induced by temperatures over 100°C. In general, it may be stated that the activity is reduced by high temperatures. This view is strengthened by the results of the time-temperature tests (Table VII and Figure 4) where it is seen that the culture loses its activity much more rapidly at higher incubation temperatures.

Tolerance of the Antibiotic to Low Temperatures

Soon after the butanol extraction method was adopted it was noted that the extracts would retain their activity for long periods of time when kept in the refrigerator at 6° to 7°C., in one case being reduced from 15 mm. to only 12 mm. in two months.

A test was run on butanol extract powder to see whether it too could be stored at 6° to 7°C. for any length of time without loss of activity. Thoroughly ground butanol extract powder was mixed with different proportions by weight of inert Dixie Clay and stored in the refrigerator. Loopfuls of the various mixtures were tested periodically for their activity. The figures given in Table XXI are averages of 8 readings.

TABLE XXI

Activity (mm.) of butanol extract
powder stored at 6 - 7°C.

	Date of testing			
	Jan. 23	Feb. 24	Aug. 1	Dec. 19
Pure powder	12	12	10	12
Powder:Clay				
1:5	9.5	9.5	9.5	9.5
1:15	9.0	9.0	9.0	9.0
1:20	8.0	8.0	9.0	10.0
1:30	8.0	8.0	8.0	8.0
1:100	6.0	5.0	6.0	6.0

The results show the stability of the substance at low temperatures. Any inconsistencies can be attributed to the difficulty in placing powdered material on test plates in a regular fashion without sometimes spreading it over too great an area. Differences in flatness of Petri dishes might also easily account for an apparent increase in activity. The test also shows the usefulness of Dixie Clay as an inert diluent and carrier.

The tolerance of the antibiotic to low temperatures was tested in another way. Lots of 25 ml. of staled .03% asparagine medium were frozen in the ice cube compartment of the refrigerator. At different times they were thawed and tested for activity, after which they were put back and frozen for another period. The figures given in Table XXII represent averages of 8 discs. They show that there is very little reduction on repeated freezing and thawing.

The antibiotic would seem to be very tolerant to low temperatures.

TABLE XXII

Tolerance of staled medium to alternate freezing and thawing

	Activity in mm.
Original staled medium	8.5
Frozen 12 hours. Thawed and tested	8.0
Replaced. Frozen 24 hours. Thawed and tested	8.0
Replaced. Frozen 6 days. Thawed and tested	7.5
Replaced. Frozen 25 days. Thawed and tested	7.0

Effect of pH on the Antibiotic

Staled .03% asparagine medium was adjusted to different pH's by adding dilute HCl or NaOH. The media were tested immediately after adjustment and after 18 and 48 hours storage at room temperature. The original staled medium had a pH of 5.89. The figures presented in Table XXIII represent averages of 8 discs.

A test on the effect of increased pH had also been run on another lot of staled medium, with the following results:

	Activity after	
pH	2 hours	18 hours
7	8 mm.	6 mm.
9.5	6.5	3.5
10.0	3.5	2.0

TEST XXIII

Effect of pH on the activity of staled medium stored at room temperature

pH	Activity (mm.) tested after 0 hours	18 hours	48 hours
2.84	7.5	4.5	trace
3.85	7.9	5.0	2.5
4.80	8.6	7.0	7.2
5.89	8.6	7.0	8.7
6.83	8.1	7.8	8.2
7.95	8.4	8.0	8.0
8.86	8.0	7.0	7.2

The results indicate that the substance in the crude state at least is intolerant to either very high or low pH's, but appears fairly stable between pH's of 5 to 8.

Effect of Dilution on the Activity of Staled Media

Table XXI shows that butanol powder remains quite active when diluted to 1:100 on the basis of weight with inert Dixie Clay. Considered on a volume basis the results are even more striking. The test confirmed previous experience in cases where it was found that a small speck of active precipitate or powder accidentally falling on a portion of the test plate gave almost as large halos as a whole loopful

of powder. This is probably due to the larger amount of powder becoming sticky and not allowing any more of the active material to diffuse out from the mass.

A test was run on the tolerance of staled .03% asparagine medium to dilution with sterile distilled water. The results, given in Table XXIV are based on averages of 8 discs.

TABLE XXIV

Tolerance of staled medium to dilution
with distilled water

% staled medium	100	90	80	70	60	50	40	30	20	10
Activity (mm.)	9.0	8.7	8.0	8.2	8.2	7.4	7.2	7.0	6.2	4.9

It will be seen from the results of Table XXIV that a considerable dilution is required before there is any appreciable loss of activity.

Discussion

Stability of different antibiotics under different conditions is quite often a characteristic feature of them. The substance from A32 seems on the basis of available data

to differ from the other 3 antibiotics (7, 17, 18) in respect to stability. Jansen and Hirschmann (17) found their sterilized cultures to retain their activity for 12 days in the dark at room temperature but to lose 90% of their activity in the light. Time-temperature production studies have shown our cultures to retain their activity at 25°C. for much longer periods, losing only about one-half of the peak activity after 60 days. Though no tests on the effect of light on the stability of A32 cultures or extracts were carried out, observations indicate that, while there is a greater loss of activity of A32 staled cultures in the light, the reduction is less marked than in the case of subtilin. Alcohol solutions of subtilin stored at 5°C. for 15 days lost all their activity against L. casei (17); butanol extracts of A32 cultures retained their activity for at least two months at 6° to 7°C. Whole cultures of the subtilin strain were found to be least affected when sterilized at pH of 2.5 (17); A32 cultures were found to be very unstable at this pH.

Data on the stability of bacillin (7) are not adequate to compare them with those for A32. The inadequacy of data on stability also makes a comparison with bacitracin (18) difficult.

COMPARISON OF A32 AND B. SUBTILIS

#231 N. R. SMITH

Subtilin production was found by Jansen and Hirschmann (17) to be very dependent upon the strain of B. subtilis. Since we had obtained a culture of the subtilin strain #231 it was desirable to make a comparison between it and A32.

The two strains when grown on P.D.A. slants under ordinary laboratory conditions are almost indistinguishable in external gross morphology. It was noted, however, that A32 sporulates much more readily on P.D.A. It is also much less adherent to the slant than is #231. Bits of vegetative growth of A32 break up much more readily in water blanks as compared with #231 which is very difficult to break up. Trace elements were found to increase subtilin production markedly (17), whereas high activity has been obtained when A32 was grown in media without them. A .3% asparagine medium was found (17) to produce good growth and subtilin activity with #231. In the case of A32 it was found that a .03% asparagine medium would produce a highly active culture though the growth was reduced.

In order to see whether #231 would grow and produce an active culture on media with an asparagine content less than .3% the following test was performed: Cultures were grown in a modified subtilin medium containing differ-

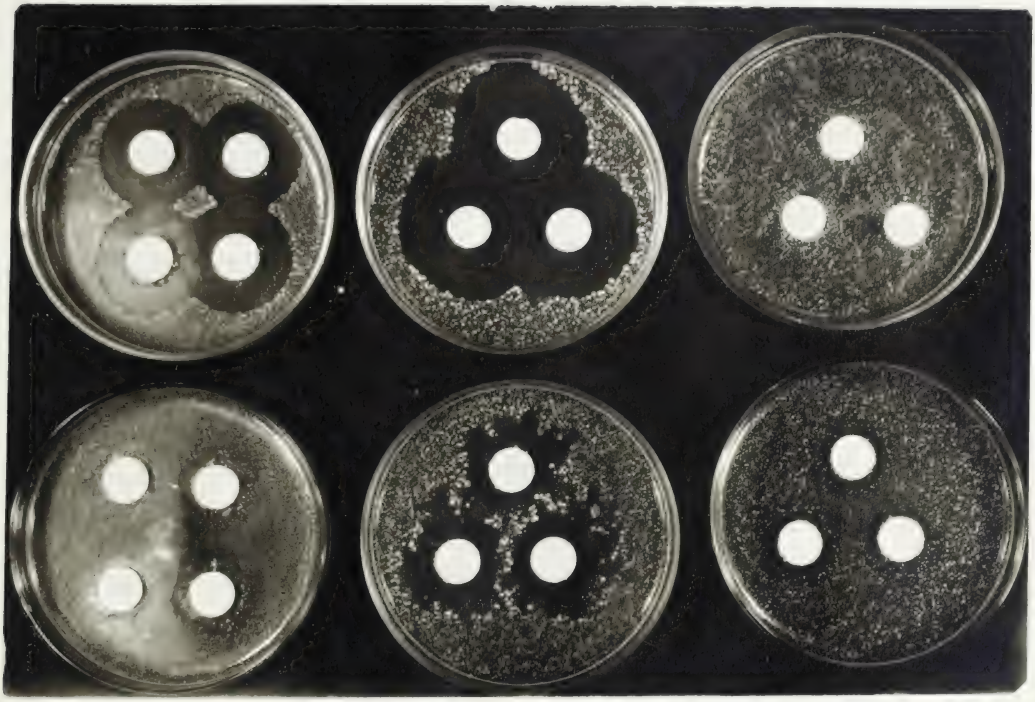

Figure 6

Relative activity as shown by halo production in Xan. 190 by preparations from B. subtilis A32 and B. subtilis #231

Top row, left to right: A32 staled medium, butanol extract, residue after extraction.
Bottom row, left to right: #231 staled medium, butanol extract, residue after extraction.

mentioned above and #231 was recovered. This may be due to the same effect found by Lewis, Dimick, and Feustel (20) in the case of B. brevis where, on relatively poor media, the pellicle was found to break up spontaneously and sink to the bottom of the flask.

Jansen and Hirschmann (17) found cultures possessing a high subtilin activity to give an intense blue color, and those with little activity no color on the addition of a few drops of a 1% alcoholic solution of $FeCl_3$ to 5 ml. of culture. Cultures of #231 grown under conditions used for A32 were found to give a blue color with $FeCl_3$. The color, though definite, was not intense. This might be due to the fact that the medium did not contain trace elements favorable for subtilin production. Throughout the course of the investigation, however, A32 never gave a blue color with $FeCl_3$. This is one definite point of difference between the subtilin strains and A32.

ACTION OF ANTIBIOTICS IN REDUCING INFECTION OF BARLEY BY XANTHOMONAS TRANSLUCENS (J. J. & R.) Dowson

The use of antibiotics in the control of plant diseases is a field still in its infancy, even though the value of microbial antagonism in the natural control of plant disease has been known to plant pathologists for several years (reviewed by 8, 34, 36). Bacterization of soil with specific antagonistic organisms has been found to result in reduced infection in a number of cases (22, 24). Bacterization of seed has also been effective in a few instances (11, 25). Parker (27) reports reduced infection from Fire Blight in pears by spraying blossoms with bacteria found antagonistic to *E. amylovora* in culture. Brown and Boyle (4) report that weak solutions of penicillin completely destroyed galls caused by *Agrobacterium tumefaciens* on *Bryophyllum* when the galls were wrapped in cotton soaked in the solution and punctured with needles pushed through the wet cotton. Rudolf (29) reports the failure of penicillin to control *Erwinia amylovora* or *Xanthomonas juglandis* infections on pear and walnut trees, respectively, by injection of the solution into the trunks, though the two pathogens were susceptible in

plate tests.* Timonin (32) found patulin to be ineffective in reducing loose smut damage to wheat, though both the spores and mycelium of U. tritici were susceptible in vitro. This is the only report of the use of an antibiotic against a seed-borne pathogen in vivo that the author is aware of.

INFECTION OF BARLEY WITH X. TRANSLUCENS

Exploratory Studies

It was necessary to find a method of infesting seed with X. translucens which would result in a high percentage of infected seedlings and which would not be too laborious. This would exclude Hagborg's (12) method whereby each seed is first smeared with the organism and then immersed in a suspension of it.

It was hoped that seed obtained in 1944 from a field at Flatbush, Alberta, showing a high proportion of

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* Since the preparation of this manuscript two other reports of the use of antibiotics against phytopathogens in vivo have come to my attention. Brown and Heep (Science 104: 208, 1946) report complete disinfection of budwood infected with Phytomonas pruni by the use of Streptomycin. Leben and Keitt (Phytopath. 37:14, 1947, Abst.) report infection of apple leaves by Venturia inequalis to be prevented or reduced by the application of an active ethanol fraction obtained from cultures of Streptomyces sp.

Bacterial Blight, would be useful without any additional treatment. This seed is hereinafter referred to as Flatbush seed. Repeated plantings in the greenhouse, however, failed to give plants showing any signs of typical water-soaked *X. translucens* lesions. This seed, sown in outside plots in 1946, gave 1 to 2% of blighted plants.

Attempts were made to infest seed artificially with *Xan. 190*, using an adaptation of Tapke's and Bever's spore-suspension method (31). Seed of Titan barley was treated for one hour with 1:320 formaldehyde, then rinsed for one-half hour in running water in order to split the hulls and loosen them around the caryopses, and then spread out to dry. Following this, it was treated with a water-suspension of *Xan. 190* for 15 minutes, after which it was put in beakers and stored for 24 hours at 25°C. in air-tight cans with wet paper towels on the bottom. The seed was then dried and sown in pots of a steam-sterilized, 3:1 soil-sand mixture. The pots were kept at 10°C. for four days, then at 20°C. for six days, after which they were placed on benches in the greenhouse. No lesions could be found on the plants after 24 days, though there was some reduction in emergence in the infested series compared with the check.

Flatbush seed was known to be of a susceptible variety and so attempts were made to infest it with *Xan. 190.* In one trial surface-sterilized seed was placed in Petri

dishes containing about 10 ml. of a bran-extract culture of Xan. 190. After several days' incubation, the seed was sown in steamed 3:1 soil. In a second trial, hulled* surface-sterilized seed was soaked for three hours in a bran-extract culture of Xan. 190, after which it was sown in steamed 3:1 soil. In neither of these experiments were any lesions found on the emerged seedlings.

An attempt at soil inoculation with Xan. 190 was also made. Xan. 190 was grown in a 10% ground barley medium which was then placed on the surface of pots of steamed soil. Flatbush seed was placed on the surface of the inoculum which was then covered with steamed soil. No lesions were observed on the resulting seedlings.

Partial vacuum methods, using Flatbush seed and Xan. 190, were then tried. Seed was placed in a nutrient broth culture of Xan. 190 in a suction flask which was then attached to a water vacuum pump for one-half hour. After treatment, the broth was poured off and the seed kept at 10°C. for five days, after which it was sown in steamed 3:1 soil. A non-hulled and an acid-hulled series were run. Readings taken two weeks after sowing showed no lesions evident, though there was a difference in emergence in the non-hulled

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* Hulled seed refers to seed with the hulls removed. Non-hulled seed refers to seed with the hull still on.

series, as follows:

Untreated check	431
Sterile broth check	389
Xan. 190 broth	354

The acid-hulled series showed lower emergence as was to be expected, but there were no differences within the series.

To see whether temperature of germination might have some effect on infection with *Xan. 190* the following test was carried out. Hand-hulled Flatbush seed was injured by scratching the embryo or endosperm with a needle. The seed was then partial-vacuum treated for one-half hour with a water-suspension of *Xan. 190*. It was then sown in 3:1 steamed soil at temperatures of 15°, 20°, 25°, and 30°C., using the greenhouse temperature tanks. Differences were noted between the germination of the various injury treatments and some slight differences were also noted between the various temperatures. No lesions were noted except for one seedling growing in the embryo-injured 30°C. series.

An experiment in which uninjured O.A.C.21 barley, hulled and non-hulled was vacuum treated with a broth culture of *Xan. 190* for one-half hour was also made. After treatment the seed was sown in small cardboard boxes containing steamed 3:1 soil. The boxes, $3\frac{1}{2}$" x $4\frac{1}{2}$" x $1\frac{1}{4}$", were made by folding and stapling at the corners a sheet of cardboard 6" x 7".

Twenty-five seeds were sown per box, and the boxes were incubated at 35°C. for four days. No lesioned seedlings were found.

Infection of Barley Using a Modified Wallin Technique

A search of the literature did not reveal any information on inoculation of seed with X. translucens using partial vacuum methods. Dr. J. R. Wallin of Iowa State College, in correspondence, recommended "placing hulled, wounded seeds in a flask of bacterial suspension, attaching a water vacuum pump to the flask for five minutes, and then allowing the seed to soak for an hour following the vacuum treatment." He also suggested trying a new isolate of X. translucens hordei or cerealis, since his best results were obtained with cerealis. This correspondence was concurrent with the publication of the technique by Wallin (39).

Since the greatest possible amount of infection was desirable, all the factors tending to give the greatest infection in Wallin's (39) study were adopted; these included soaking the seed for 24 hours after treatment and germinating them at 35°C. Since our tests using a half-hour vacuum treatment produced no infection, the treatment was increased to one hour. Seeds were sterilized for 15 minutes with a

0.3% solution of calcium hypochlorite before treatment. The inoculum used was a water suspension of X. translucens f. sp. cerealis obtained from Dr. W. A.F. Hagborg and hereinafter referred to as Xan. 1015. The treated seeds were sown in 3:1 steamed soil in the aforementioned cardboard boxes at the rate of 25 seeds per box, and germinated at 35°C. Examination of the seedlings five days later revealed a very high percentage of seedlings showing typical water-soaked lesions. Later trials showed 30°C. to be a satisfactory germination temperature, and the use of a 1:1 steamed soil largely eliminated crusting of the soil surface. For all subsequent work the modified Wallin technique described above was adopted and, since Xan. 1015 gave such good results, it was the pathogen adopted except where otherwise noted. Xan. 190 was, however, retained as the test organism for plate tests because of its quick growing ability and the production of sharp, easy-to-measure halos. Xan. 190 differs from the other Xanthomonas translucens strains in colony appearance on P.D.A. slants in having at first a creamy white color, after which golden yellow streaks appear in the colony which soon after assumes the typical golden color. All the other strains show the typical color from the beginning.

In view of the success obtained using the modified Wallin technique it is rather difficult to account for the previous negative results. Leaf inoculations had shown Xan.

190 to be pathogenic though perhaps not as pathogenic as *Xan. 1017*, the other culture of *hordei-avenae* available. That the reason for failure was not entirely pathogenicity was shown by the following experiment. Using the modified Wallin technique, O.A.C.21 barley was infested with *Xan. 190* and three other strains. One hundred seeds were used per treatment. The results are presented below.

Inoculum	No. of seedlings emerged	No. of lesioned seedlings
Xan. 190	64	19
Xan. 1017	46	38
Xan. 1016	49	43
Xan. 1018	57	50

Though it is evident from both the number of emerged and lesioned seedlings that *Xan. 190* is not as pathogenic as are the other strains, it is reasonable to assume that some lesioned seedlings should have appeared with the earlier vacuum methods. Failure to do so is probably attributable to the shorter one-half-hour treatment and the absence of soaking for 24 hours after treatment. Drying of the seed after treatment in the earlier methods probably had no effect, as will be seen from subsequent data.

EFFECT OF A32 STALED MEDIA ON INFECTION OF BARLEY WITH X. TRANSLUCENS

To see whether staled .03% asparagine medium had any effect on infection of O.A.C.21 barley with Xan. 1015, staled medium was added to the infestation medium and the seed was then infested using the modified Wallin technique. One hundred seeds were used per treatment. The results are presented in Table XXV.

TABLE XXV

Effect of staled A32 medium on the ability of Xan. 1015 to infect barley seedlings

Treatment	No. of seedlings emerged	No. of lesioned seedlings
100 ml. distilled H_2O	34	0
100 ml. distilled H_2O + 20 ml. staled medium	75	0
100 ml. Xan.1015 suspension	36	6
100 ml. Xan.1015 suspension + 20 ml. staled medium	47	15

It would appear from the infection data that staled medium seemed to increase infection. The increase in emergence, however, must be considered in the disease picture.

Wallin (39) found X. translucens cerealis to decrease germination of barley, especially at higher temperatures. This is confirmed by our results. The foregoing results also strikingly confirm the non-phytotoxic property of A32. This is especially marked since the particular lot of seed used had the embryo quite badly injured, and A32, if phytotoxic, would have had particularly favorable seed to work on. A32 seems to have a protective effect.

In a second experiment care was taken not to injure the embryo too much by scratching it on the side rather than directly across the face of the coleoptile. Two varieties, O.A.C.21 and White Hulless, were used. To 100 ml. of a water suspension of Xan. 1015, 25 ml. of staled medium were added. The seed was then infested using the modified Wallin technique and 100 seeds per treatment. The results are presented in Table XXVI.

TABLE XXVI

Effect of staled A32 medium on the ability of Xan. 1015 to infect barley seedlings

Treatment	No. of seedlings emerged	No. of lesioned seedlings	Disease rating
O.A.C.21			
Xan. 1015 + A32	70	19	29
Xan. 1015	51	14	43
White Hulless			
Xan. 1015 + A32	73	21	28
Xan. 1015	46	5	39

In this experiment again there is seen the beneficial effect of A32 on the emergence in both varieties. In order to evaluate the effect of increased emergence in the disease picture a somewhat arbitrary method was adopted to arrive at a disease rating. A figure of 80 was taken as the normal germinability of uninfested damaged seed. The same lot of seed was used throughout the investigation and it will be seen from this and other experiments that 80 is not too high a figure. All emerged seedlings not showing lesions were considered as healthy. The number of healthy seedlings was taken from 80 to give the number of "diseased" seedlings and this figure was used as the disease rating. Using the first row of figures in Table XXVI the disease rating was arrived at as follows:

No. of seedlings emerged 70
No. of lesioned seedlings 19

No. of healthy seedlings 51

Normal germination 80
No. of healthy seedlings 51

Disease rating 29

On this basis it can be seen that A32 had a marked effect in reducing the infection in both varieties.

It was desirable to know whether seed infested with *Xan. 1015* and then dried would still produce lesioned seedlings. This was particularly desired, since seed sown in the soaked-up condition after infection is very susceptible to molds etc., especially at 30°C. O.A.C.21 seed was

subjected to the modified Wallin technique using 100 ml. of water plus 25 ml. staled medium, 100 ml. of water suspension of _Xan. 1015_, and 100 ml. of a suspension of _Xan. 1015_ plus 25 ml. of staled medium. One series was sown immediately after infection and the second series was dried for one week before being sown. One hundred seeds were used per treatment. The results are presented in Table XXVII

TABLE XXVII

Effect of staled A32 medium on the infection of barley with _Xan. 1015_ sown wet or dry

	Sown wet			Sown dry		
Treatment	No. of seedlings emerged	No. showing lesions	Disease rating	No. of seedlings emerged	No. showing lesions	Disease rating
A32	78	0	2*	80	0	0
Xan. 1015	31	12	61	47	15	48
Xan.1015 + A32	73	39	46	74	26	32

* Not really disease but appears as such owing to method of arriving at disease rating.

The results in Table XXVII show first that seed dried after infestation will still produce lesioned seedlings. Such pre-infested seed is obviously more satisfactory for experiments on control of the disease. The beneficial effects

of A32 medium on the disease rating are evident in both the wet- and dry-sown series.

It had been noted in a previous test that barley seed vacuum-treated with n-butanol for five minutes and then allowed to soak for 15 minutes, would not germinate. Use of butanol extract in this manner on pre-infested seed would be precluded as a control method. Pre-infested seed was subjected to various treatments in an attempt to control the disease. The treatments were as follows:

1. Vacuum treated 1 hour in undiluted A32 staled .03% asparagine medium, then allowed to soak for 14 hours. Sown in steamed 1:1 soil.

2. Moistened seed was treated with 1:15 butanol-extract-Dixie-Clay powder (Table XXI). The seed was completely covered and the excess then shaken off. Sown in 1:1 steamed soil.

3. Seed was sown in soil previously treated as follows: columns of sterile dry 1:1 soil had A32 staled .03% asparagine medium run through them several times, after which soil was mixed half and half with more steamed 1:1 soil.

4. Seed was sown directly in unsterile 1:1 soil.

5. Seed was sown directly in sterile 1:1 soil (untreated check).

One hundred and fifty seeds were sown per treatment. The results are presented in Table XXVIII.

TABLE XXVIII

Effect of various treatments on barley pre-infested with *Xan. 1015*

Treatment number	Number of seedlings emerged	Number of seedlings lesioned	% disease*	% emergence
1	66	16	24.2	44.0
2	89	27	30.3	59.3
3	73	11	15.1	48.7
4	38	5	13.1	25.3
5 (check)	88	34	38.6	58.7

$$* \ \% \text{ disease} = \frac{\text{number of lesioned seedlings}}{\text{number of seedlings emerged}} \times 100$$

The results are suggestive of certain lines of investigation that might be followed up. The 1:15 butanol-extract-Dixie-Clay powder appeared to reduce infection as did the soil treated with staled medium. Limitations of time did not allow any further tests to be carried out. Such tests should include studies on how long the active material is retained in sterile and unsterile soil, and also tests with other dilutions of butanol-extract powder.

EFFECT OF SUBTILIN ON INFECTION OF BARLEY BY *X. TRANSLUCENS**

While work was proceeding on the effect of A32 in

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* The experiments using subtilin in the control of infection have been published (10) with the approval of the Committee on Graduate Studies, University of Alberta.

reducing infection, it seemed desirable to test subtilin, to which X. translucens is susceptible, in this regard.

In a preliminary experiment O.A.C.21 barley was infested with a suspension of Xan. 1015 in a solution of 1:1000 subtilin in distilled water. The following results were obtained:

Treatment	Seedlings emerged	Seedlings lesioned
Distilled water	72	0
Xan. 1015 in water	52	26
Xan. 1015 in 1:1000 subtilin	76	0

A more extensive experiment was undertaken using different dilutions of subtilin. The treated seed was sown in 1:1 soil in 6-inch flower pots. Twenty-five seeds per pot and 200 seeds were used per treatment. One series was sown in steamed soil and another series in unsterile soil. Two hundred seeds were sown per treatment. The pots were kept at 30°C. for 10 days after which notes were taken on the number of emerged and lesioned seedlings. The results are presented in Table XXIX. The pots were then left on a greenhouse bench for 10 days longer, when a second count was made. No increase in emergence occurred in the sterile series, but there was some increase in the unsterile series. The second disease count gave results comparable to the first.

TABLE XXIX

Effect of different dilutions of subtilin on the ability of Xan. 1015 to infect barley seedlings

Xan. 1015 in dilution of	No. plants emerged	No. showing lesions	% disease*
		Sterilized Soil	
1:1000 subtilin	120	1	0.8
1:3000 "	127	39	30.7
1:5000 "	124	52	41.9
Sterile distilled H_2O	83	39	47.0
		Unsterilized Soil	
1:1000 subtilin	46	0	0.0
1:3000 "	44	3	6.8
1:5000 "	59	15	25.4
Sterile distilled H_2O	49	10	20.4

$$* \ \% \text{ disease} = \frac{\text{Number of seedlings showing lesions}}{\text{Number of seedlings emerged}} \times 100$$

It will be noted from the above table that 1:1000 subtilin was almost completely effective in preventing infection by Xan. 1015 when applied simultaneously with the pathogen. Comparable infection trends were obtained in sterilized and unsterilized soil, though the percentages of infection are consistently lower in the unsterilized series. This is probably attributable to the action of the natural soil microflora, as has been reported for certain diseases of flax (14). The natural flora is also probably responsible for the low degree of emergence in the unsterilized series,

since the seed was sown in a soaked condition and kept at 30°C., which would make it susceptible to such action. The infection ratings in this test were based on the number of emerged seedlings. Had the reduction in emergence been taken into account,, as in the experiments using A32 staled medium, the results would have been still more striking.

It was desirable to know whether subtilin would also be effective when used on pre-infested seed. Such seed was treated with 1:1000 subtilin by two methods:

1. Under partial vacuum for 1 hour and then allowed to soak in the solution for 20 to 24 hours. This is the same procedure as is used in infestation.

2. Under partial vacuum for 15 minutes. The treated seed, together with an untreated check, was then sown at the rate of 350 seeds per treatment in steamed 1:1 soil and kept at 30°C. Emergence and disease counts taken seven days later are presented in Table XXX.

The results in Table XXX show that subtilin will reduce infection in pre-infested seed. The low emergence in the first treatment is probably due to the effect of two rather severe vacuum treatments, one during the infestation and the other in the application of subtilin.

TABLE XXX

Effect of treating pre-infested barley seed with a 1:1000 dilution of subtilin

Treatment	Seedlings emerged	Seedlings lesioned	% disease*	% emergence
Vacuum treated 1 hour soaked 20-24 hours	165	29	17.6	47.1
Vacuum treated 15 min.	261	90	34.5	74.6
Check	249	103	41.4	71.1

$$* \% \text{ disease} = \frac{\text{Number of lesioned seedlings}}{\text{Number of seedlings emerged}} \times 100$$

GENERAL CONCLUSIONS AND DISCUSSION

The work thus far carried on would seem to indicate that the antibiotic from A32 differs, in some respects at least, from antibiotic substances isolated from other strains of B. subtilis. It differs from subtilin (17) in several marked respects. Subtilin is found mainly within the cells of the organism and not in the cell-free filtrate as is the case with the antibiotic from A32. Subtilin is much more unstable than the substance from A32. A strain of B. subtilis #231, which is a known subtilin strain, has always produced a blue color reaction both under the conditions used by Jansen and Hirschmann (17) and under those obtaining in this investigation. On the other hand, cultures of A32 have never, in the course of this work, given this color reaction. Moreover, the method of isolation of subtilin has not given satisfactory results with the antibiotic from A32.

Because of limited data it is more difficult to evaluate differences between the antibiotic from A32 and bacillin (7) or bacitracin (18). Both of these, like the substance from A32, are found mainly in the cell-free filtrate. Bacillin is removable from the culture filtrate by activated charcoal. Unlike the substance from A32, however, it can be removed from the charcoal by ethanol or methanol. Bacitracin can be extracted from the medium by n-butanol as can the

substance from A32. Bacitracin is found to produce its maximum titre after 3 to 5 days at 37°C. Time-temperature studies in the case of A32 show its maximum activity to be reached in about 10 days.

The use of antibacterial spectra in the differentiation of antibiotics is not always very reliable when trying to compare the results of different investigations. Just as the formation of antibiotics is dependent upon strain, so is the susceptibility or resistance of a given test organism dependent upon strain. The test organisms used by the different investigators must be of the same strain in order that comparisons may be reliable. The use of different test media by different investigators also makes comparisons questionable. This is well illustrated in the case of bacillin, where Foster and Woodruff (7) find the presence of complex natural materials in the test agar to reduce greatly or abolish the antibacterial properties of the antibiotic. It is also illustrated in a small way by our experience with Aerobacillus polymyxa and the substance from A32: tested on nutrient agar, no or negligible halos were obtained, whereas on potato-dextrose agar considerable activity was evident. Antibacterial spectra, to be of much use in establishing the identity of antibiotics from the same species, should be carried out using identical strains of test organisms. A variety of methods of testing, as well as a variety of media, should be employed.

As previously suggested, it may quite possibly be that several substances contribute to the antibiotic effect of A32 cultures as has been suggested for subtilin (17). Foster and Woodruff also suggest (7) that their bacillin strain of B. subtilis produces two distinct substances, depending on the culture medium used. It may easily be that the antibiotics from different strains of a species contain certain substances in common, or that different antibiotics contain different proportions of the same substances.

The substance thus far obtained from A32 is not in a pure form. Further work of a chemical nature, especially on the butanol extract which contains all the activity of staled media, is necessary. Detailed studies on the probable effect of trace elements, or submerged aerated culture techniques on the production of the antibiotic are also desirable.

This investigation was undertaken with the purpose in view of finding a substance of practical value for the treatment of seed. The selection of A32 as the antagonist to work with was based partially on its effect on the seed-borne phytopathogen, X. translucens. In the antibacterial tests it proved to be effective under the conditions used against a number of phytopathogens including several other seed-borne ones.

Use of the antibiotic from A32 in experiments on the control of Bacterial Blight due to X. translucens was

held up owing to the failure of all methods to give successful infection of seed. The use of the modified Wallin technique overcame this difficulty. The results so far obtained are suggestive. Crude filtrates from A32 were effective in reducing damage due to Bacterial Blight and, in one case, subtilin was found to prevent the disease from appearing at all. It must be remembered that, in the case of A32, unpurified or at least relatively crude material was used. The results with A32 are thus perhaps even more suggestive than those using subtilin. It is not implied that the results are immediately applicable in the treatment of seed, for it is realized that the method used was a highly artificial one. The results do indicate, however, that with future refinements of technique, both in production and application, seed treatment with antibiotics may have practical possibilities. The use of methods such as were used in this investigation would seem, however, to provide results that are more reliable for assessing the practical value of antibiotics against seed-borne bacterial (and perhaps fungal) pathogens than those obtained from plate tests alone.

Use of antibiotics in the field of plant disease has, it would appear, definite possibilities. It is not at all unlikely that success so far obtained by the use of antagonistic organisms in the control of plant diseases has been due, in considerable measure, to the antibiotic substances produced by these organisms.

In the field of medicine the use of antibiotics as therapeutic agents is a relatively new development. The progress made up to the present has been made under the stimulus of war and also because a single human or animal is quite valuable. The progress, however, has been due to a great deal of research, whole laboratories being engaged on such work as the finding of better producing strains, better methods of isolation and purification or clinical use, etc. In the plant disease field the use of antibiotics is in an early stage. There are a great many difficulties to be overcome and we should not expect that they will be overcome without a great deal of research.

Some of the problems to be considered are these: the antibiotic must not be toxic; it must not lose its effectiveness when in contact with plants, with microorganisms, or with the soil; it must be stable under a wide range of conditions of temperature, moisture, aeration, reaction, and many other factors. The cost factor might be for a long time more limiting than in animal diseases. The possibility of finding non-toxic or poisonous materials among the antibiotics is much greater than among the materials now in use to control plant diseases. There is always too the possibility of combining the specificities of several antibiotics into one treatment.

Though the whole field is yet in its infancy, the reports beginning to appear in the recent literature augur

well for its future.

SUMMARY

1. This investigation was undertaken with a view to finding a substance of practical value for the treatment of seed. Forty antagonistic microorganisms, including bacteria, Actinomycetes, and Penicillia, were isolated from the soil.

2. One of the antagonists, A32, was chosen for study on the basis of its non-phytotoxic property and its antagonism to *Xanthomonas translucens* (*Xan. 190*).

3. A32 was identified as a typical rough strain of *Bacillus subtilis*.

4. The agar-plate paper-disc method, using *Xan. 190* as the test organism, was adopted for assay purposes.

5. A32 was found to grow and produce cultures with antibiotic activity on a number of media. The medium finally adopted was a modified subtilin medium containing .03% asparagine.

6. The amount of exposed surface area of medium was found to be important in the production of the antibiotic with shallow layers giving the best results.

7. An incubation period of 6 to 10 days and an incubation temperature of 30°C. were found to be optimum for the production of the antibiotic.

8. The activity of cultures was retained much longer at lower than at higher incubation temperatures.

9. The antibiotic is elaborated in the early part of the growth of the cultures and is not a product of cell breakdown.

10. Accumulation of end products does not seem to be a factor in decreasing the activity of cultures. Prolonged exposure to elevated temperatures appears to be a chief factor of inactivation.

11. Exploratory methods of extraction of the antibiotic are described. An n-butanol extraction method was finally adopted.

12. The antibiotic was adsorbed very well by activated alumina and charcoal and by various soils. No satisfactory method for its elution from the adsorbents was found.

13. The antibiotic proved active in plate tests against a number of bacteria including several phytopathogens.

14. The antibiotic from A32 and subtilin were both found to be bacteriostatic in their action against Xan. 190.

15. Several factors were found to affect the width of halo produced in the paper-disc method of assay. The nature of the seed suspension and the time elapsing between the preparation of the plate and the application of the antibiotic were the most important of these. Experience has shown the depth of the agar in the plate to be a factor as well.

16. The antibiotic is relatively thermostable at temperatures below 100°C. but less so at temperatures over 100°C. It was found to be stable for long periods at 6° to 7°C. as well as when frozen in the crude state. It was shown to be most stable at a pH of 5 to 8, and was also shown to be relatively tolerant to dilution.

17. A32 was found to differ in several respects from the subtilin strain of *B. subtilis* #231.

18. The antibiotic from A32 differs from subtilin in several respects.

19. A modified Wallin technique proved to be a satisfactory method of inoculating barley seed with *X. translucens* for the production of Bacterial Stripe in the seedlings.

20. The severity of Bacterial Stripe infection was reduced by treatment of seed with media staled by A32 applied either during the seed inoculation process or to seed previously infested with *X. translucens*.

21. Pure subtilin in sterile distilled water applied to seed in a similar manner was also effective in reducing Bacterial Stripe infection.

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